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Description

The present invention relates to biologically useful particles. In particular it relates to modified particles derived from the yeast retrotransposon Ty. Particles formed from such proteins are immunogenic and can be used in immunotherapeutic or prophylactic vaccines or as diagnostic agents.

An ideal immunogen is a polymer of multiple antigen determinants assembled into a high molecular weight, particulate complex. A substantial disadvantage of most antigens produced by recombinant DNA techniques for vaccines is that they are usually produced as simple monomeric proteins. This is not the ideal configuration of an immunising antigen as it does not readily permit the cross-linking of the components of the immune system. Such crosslinking is required for maximum stimulations of humoral and cellular immunity. For these reasons it would be advantageous to develop polyvalent, particulate carrier systems for immunising antigens.

WO-A-8803562 and WO-A-8803563 describe the use of certain fusion proteins derived from retrotransposons or RNA retroviruses for pharmaceutical, diagnostic or purification applications. Such particles are designated virus-like particles (VLPs) when derived from the yeast retrotransposon Ty. The above published PCT applications note that polyvalent particles are useful for immunisation purposes because their polyvalent nature provides that more antibodies will be raised against the particulate antigens used. The particles are formed of fusion proteins having a particle-forming sequence and, in some embodiments at least, an antigenic sequence. In the examples, the antigenic sequence is positioned C-terminal to the particle-forming sequence.

While the above approach is promising, a potential difficulty is that insertion of the antigen at the C-terminal end of the particle-forming protein may not in all cases be optimal for presentation to the immune system. Animals immunised with recombinant VLPs may elicit a higher titre response to the Ty component than to the added antigen. It would therefore be highly advantageous to construct antigen-presenting particles where the antibody response to the added antigen is augmented. Such particles might also have enhanced ability to stimulate a cell-mediated immune response, such as a T-cell response, a Cytotoxic T-lymphocyte (CTL) reponse or a Natural Killer (NK) cell response. It would further be advantageous if, following immunisation with such particles, the antibody response to the particle-forming moiety was reduced or preferably prevented.

One way to improve the presentation of the antigenic sequence to the immune system might be to insert the antigenic sequence of interest within the particle-forming sequence. However, correct insertion of the antigenic site within the particle-forming protein is likely to be critical for particle formation. Insertions might disrupt the secondary and tertiary structure determinants of the monomer, or the quaternary interactions between monomers necessary for particle formation.

One approach to deduce suitable surface-exposed insertion sequences has been to use the understanding of the three-dimensional structure of viruses elucidated by X-ray crystallography. Such precise analysis of the structure of the polio virus has enabled particulate chimaeric proteins to be created whereby heterologous antigenic sequences are substituted for amino-acids present in the suface-exposed epitopes of this virus (Dedieu *et al.*, J. Virol. (1992) 66 3161-3167; Burke *et al*, Nature (1988) 332 81-82; Evans *et al.*, Nature (1989) 339 385-388). However, these polio virus constructions are limited by the need to produce a viable virus; even some very short sequences cannot be tolerated.

Detailed analysis as described for poliovirus is not possible for proteins which have not yet been crystallised. Where particles have a well-characterised tertiary β-barrel structure, internal insertions of heterologous antigenic sequences into presumed surface exposed regions have been made using predictive models based on sequence alignment. For example, hybrid particles prepared from the hepatitis B core antigen and an antigen derived from a virus with an analogous secondary structure were found to maintain particle formation and enhance the immunogenicity of the inserted antigen (Schodel *et al.*, J. Virol. (1992) 66 106-114; Brown *et al.*, Vaccine 1991 9 595-601). Substitutions of heterologous peptides into presumed surface-exposed, immunodominant regions of the hepatitis B surface antigen also gave rise to particulate, chimaeric proteins with enhanced immunogenicity (von Brunn *et al.*, Vaccine 1991 9 477-601), although considerable amounts of lipid were found to be associated.

However, retrotransposons have a very poorly understood structure and it is not currently believed that they possess a β-barrel (Burns *et al.*, J. Mol. Biol. (1990) 261 207-211). Suitable sites for insertion of antigens into these particulate proteins are therefore not known or predictable. In retroviruses (which have a very similar structure to retrotransposons) it has been shown that insertion of an antigen into the middle of the gag sequence destroys the particle-forming nature of this sequence (Luo *et al.*, Proc Natl. Acad. Sci. USA 89 10527-10531 (1992)).

The present inventors have identified the surface-exposed immunodominant epitopes within the yeast retrotransposon Ty p1. Immunogenic sites are not necessarily surface exposed; high titre antibodies are frequently elicited against core proteins during viral infections even though such proteins are not exposed on the surface of the particle (eg the influenza nucleoprotein). The inventors have also found that insertion of heterologous antigenic sequences into such epitopes does not prevent particle formation. In retrotransposons the size of insertion which can be tolerated without disrupting particle formation appears to be remarkably large; much greater than has been described for any other system, where generally substitutions have been preferred. The resulting hybrid particles exhibit reduced immuno-

genicity of the particle forming protein, and an enhanced immune reponse to the inserted sequence.

According to a first aspect of the invention, there is provided a non-natural particle-forming protein comprising a first self-assembling particle forming amino acid sequence substantially homologous with a yeast retrotransposon Ty p1 protein and a second amino acid sequence, wherein the second sequence is antigenic and is incorporated within an epitope of the first amino acid sequence, which epitope, on particles formed from the first amino-acid sequence alone, is surface-exposed.

Such constructions may be produced either by insertion of antigenic sequences into these surface epitopes to form true hybrid proteins, or by substitution of the native amino acids found at such sites with the amino acid sequence of interest, or by a combination of deletion, substitution and insertion.

The surface-expressed epitopes will generally be found in the N-terminal half of the first particle forming protein, the sequence of which is disclosed in Dobson *et al.,* 1984 EMBO J. 3 1115. In particular, three consensus surface-exposed regions have been identified in the N-terminal half of the particle-forming protein p1 of the retrotransposon Ty, located at amino acids 2142 (position A), amino acids 55-74 (position B) and amino acids 93-142 (position C) as shown in Figure 1 and summarised in Table 1. Proteins in which the second amino acid sequence is located within at least one of these regions in the first amino acid sequence are preferred. Within these regions, any suitable insertion site may be chosen for the second sequence. These sites include those between amino-acids 30-31, 67-68, 113-114 and 132-133 of the Ty protein and have been designated sites A, B, C_1 and C_2 respectively, but other sites are equally appropriate.

Particles derived from Ty may have advantages over those derived from polio or Hepatitis for use as vaccines. Pre-exposure to hepatitis or polio vaccines can compromise an effective subsequent reponse against the chimaera. The use of particles derived from Ty is therefore preferable, as there will be less likelihood of a patient having a pre-existing immunological response. Since Ty is not a pathogen, vaccination with Ty will not cause exposure to pathogenic antigens.

The expression "substantially homologous", when describing the relationship of an amino acid sequence to a natural protein, means that the amino acid sequence can be identical to the natural protein or can be an effective but truncated or otherwise modified form of the natural protein or can share at least 50%, 60%, 70%, 80%, 90%, 95% or 99%, in increasing order of preference, of the residues of the natural protein or its modified form. "Effective" means that the particle forming ability of the natural protein is retained (or at least not substantially lost). Alternatively or in addition, a nucleic acid sequence encoding the amino acid sequence may hybridise, for example under stringent conditions, to a nucleic acid sequence encoding the natural protein or its truncated form, or would do so but for the degeneracy of the genetic code. Stringent hybridisation conditions are known and are exemplified by approximately 0.9 molar salt concentration at approximately 35° to 65°C.

The antigenic sequence may correspond to a sequence derived from or associated with an aetiological agent or a tumour. The aetiological agent may be a microorganism such as a virus, bacterium, fungus or parasite. The virus may be: a retrovirus, such as HIV-1, HIV-2, HTLV-I, HTLV-II, HTLV-III, SIV, BIV, LAV, ELAV, CIAV, murine leukaemia virus, Moloney murine leukaemia virus, and feline leukaemia virus; an orthomyxovirus, such as influenza A or B; a paramyxovirus, such as parainfluenza virus, mumps, measles, RSV and Sendai virus; a papovavirus, such as HPV; an arenavirus, such as LCMV of humans or mice; a hepadnavirus, such as Hepatitis B virus; a herpes virus, such as HSV, VZV, CMV, or EBV. The tumour-associated or derived antigen may for example be a proteinaceous human tumour antigen, such as a melanoma-associated antigen, or an epithelial-tumour associated antigen such as from breast or colon carcinoma.

The antigenic sequence may be also derived from a bacterium, such as of the genus *Neisseria, Campilobacter, Bordetella, Listeria, Mycobacteria* or *Leishmania,* or a parasite, such as from the genus *Plasmodium,* especially P. *falciparum,* or from a fungus, such as from the genus *Candida, Aspergillus, Cryptococcus, Histoplasma* or *Blastomyces.*

The antigenic sequence may typically vary in length from between 6 and 60 amino acids, for example 6-50, 6-40, or 6-30, although it is not possible with precision to give universally appropriate maxima and minima. The sequence should be sufficiently long to give rise to the desired immunogenic response, but not so long as to cause unacceptable distortion to the rest of the molecule.

Preferred antigenic sequences are antigenic sequences corresponding to epitopes from a retrovirus, a paramyxovirus, an arenavirus or a hepadna virus, or a from human tumour cell. Examples include known epitopes from:

1) HIV (particularly HIV-1) gp120,
2) HIV (particularly HIV-1) p24,
3) Influenza virus nucleoprotein and haemagglutinin,
4) LCMV nucleoprotein,
5) HPV L1, L2, E4, E6 and E7 proteins,
6) p97 melanoma associated antigen,
7) GA 733-2 epithelial tumour-associated antigen,

8) MUC-1 epithelial tumour-associated antigen,
9) Mycobacterium p6,
10) Malaria CSP or RESA antigens,
11) VZV gpI, gpII or gpIII

Particularly preferred antigenic sequences comprise a sequence substantially homologous with an antigenic portion of the third variable domain of a lentivirus. This region, known as the V3 loop or GPGR loop is found between amino acids 300 and 330 of the envelope glycoprotein gp120 of HIV-1 and in analogous positions of other lentiviruses. The V3 loop is defined by two flanking cysteine residues linked by a disulphide bond and, for HIV-1 at least, is the major neutralising epitope of the virus (Putney *et al* 1986 *Science* **234**, 1392; Rusche *et al* 1988 *Proc. Natl. Acad. Sci.* **85**, 3198; Palker *et al* 1988 *Proc. Natl. Acad. Sci.* **85** 1932; and Goudsmit *et al* 1988 *AIDS* **2** 157). The antigenic portion of choice may constitute the whole or half of the V3 loop. However, a conserved sequence of the V3 loop may be useful in conferring immunity against more than one isolate of a virus (such as HIV-1).

A number of isolates of HIV-1, in which the sequence of the V3 loop varies from isolate to isolate, are known. The most common isolates are HXBII, RF and MN; MAL, ELI and BH10 are also important, but the MN isolate may be the most clinically relevant. Laboratory isolate IIIB is a mixture of strains BH10 and HXBII. The invention is not limited to sequences derived from the V3 loop of any particular isolate, some of which are shown below.

BH10	SNCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCNISG
HXBII	SNCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNISG
MN	SNCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNISG
MAL	SNCTRPGNNTRRGIHFGPGQALYTTGIVDIRRAYCTING
RF	SNCTRPNNNTRKSITKGPGRVIYATGQIIGDIRAHCNLSGS
ELI	STCARPYQNTRQRTPIGLGQSLYTTRSRSIIGQAHCNISG.

Neither is the invention limited to natural V3 loop sequences. Examples of variant V3 loop sequences which can be used in the invention include:

MAL(var)	SNCTRPGNNTRRGIHFGPGQALYTTGIVDEIRRAYCNISG
RF(var)	SNCTRPNNNTRKSITKQRGPGRVLYATGQIIGDIRKAHCNSIG
ELI(var)	STCARPYQNTRQRTPIGLGQSLYTTRGRTKIIGQAHCNISG.

A comparison of the sequences of the V3 loop from many different HIV-1 isolates shows great variation between isolates. Antibodies raised against the V3 loop are therefore usually type-specific. However, approximately 60% of isolates to date have the consensus sequence GPGRAF, and more than 80% have a GPGR sequence at the tip of the loop. Recent studies have shown that immunisation with peptides containing the GPGRAF consensus sequence or cross-immunisation with recombinant gp120 from different isolates can induce antibodies which cross react between isolates. The GPGRAF consensus sequence may itself be used in the invention.

Other embodiments of the invention involve the use of short sequences of V3 which are not necessarily conserved between various isolates. Whatever V3-derived or V3-related sequence is used, the resulting fusion proteins, or at least particles assembled from them, will be similar antigenically to natural V3 loop sequences in the sense that they cross-react with one or more common antibodies.

More than one V3-derived sequence can be present in a fusion protein of the invention. This embodiment may enable a single fusion protein to be useful in the protection against more than one HIV isolate: therefore, V3-derived sequences from different HIV isolates can be present on the same molecule.

More generally, it will be appreciated that the invention provides considerable flexibility in the nature of the antigenic, second amino acid sequences and the way in which they (if there are more than one) can be located within the first amino acid sequence. For example, two or more identical second amino acid sequences can be inserted in tandem into the same insertion site, two or more identical second amino acid sequences can be inserted into different insertion sites, two or more different second amino acid sequences can be inserted in different insertion sites (or even a single insertion site), and it will be appreciated that the two or more different amino acid sequences may be derived from different epitopes of the same antigen.

As fusion proteins in accordance with the invention spontaneously assemble into particles, it is possible by means of the invention to prepare multivalent particles.

According to a second aspect of the invention, there is provided a particle comprising a plurality of non-natural proteins as described above. Particles in accordance with the invention may contain a heterologous, or, preferably, homologous population of proteins. Each protein may have any of the configurations described above.

According to a third aspect of the invention, there is provided nucleic acid (particularly DNA) coding for a fusion protein as described above. It will generally be the case that the nucleic acid will be capable of being expressed without splicing or anti-termination events. There will generally be no frame shifting, but frame shifting is not necessarily always excluded.

Further according to the present invention is provided a vector including nucleic acid as described above.

Expression vectors in accordance with the invention will usually contain a promoter. The nature of the promoter will depend upon the intended host expression cell. For yeast, *PGK* is a preferred promoter, but any other suitable promoter may be used if necessary or desirable. Examples include *GAPD*, *GAL*1-10, *PH05*, *ADH*1, *CYC*1, Ty delta sequence, *PYK* and hybrid promoters made from components from more than one promoter (such as those listed). For insect cells, preferred promoters are the polyhedrin and p10 promoters from *Autographica californica* nuclear polyhedrosis virus (AcNPV). Those skilled in the art will be able to determine other appropriate promoters adapted for expression in these or other cells. Vectors not including promoters may be useful as cloning vectors, rather than expression vectors.

The invention also includes host cells, for examples bacterial cells, such as *E. coli,* which may be used for genetic manipulation, yeast cells such as *Saccharomyces cerevisiae* or *Pichia pastoris* or animal cells.

The augmented immunogenic nature of the particles in accordance with the invention, facilitates the production of antibodies with specific characteristics. The invention thus further provides antibodies raised or directed against particulate antigens of the invention; such antibodies may be polyclonal or monoclonal. For the production of human monoclonal antibodies, hybridoma cells may be prepared by fusing spleen cells from an immunised animal with a tumour cell. Appropriately secreting hybridoma cells may thereafter be selected.

Particulate antigens in accordance with the invention may be used in the preparation of vaccines, for example immunotherapeutic vaccines, which form a further aspect of the invention. The vaccine may comprise a particulate antigen and a physiologically acceptable non-toxic carrier, such as sterile physiological saline or sterile PBS. Sterility will generally be essential for parenterally administrable vaccines. One or more appropriate adjuvants may also be present, but are not always necessary. Examples of suitable adjuvants include muramyl dipeptide compounds such as prototype muramyl dipeptide, aluminium hydroxide and saponin.

Vaccines in accordance with the invention may present more than one antigen. Either a cocktail of different particulate antigens may be used, or a homogeneous population of particulate antigens having more than one epitope could be used, as described above. It may in practice be simpler for a vaccine to contain a mixture of different particulate antigens.

Fusion protein and particulate antigens of this invention are useful as diagnostic reagents. Particulate antigens for diagnostic purposes are particularly advantageous because they can be physically separated by centrifugation or filtration and can be directly dispersed on solid supports such as glass or plastic slides, dip sticks, macro or micro beads, test tubes, wells of microtitre plates and the like. The particulate antigens of this invention may also be dispersed in fibrous or bibulous materials such as absorbent disk (US-A-4,632,901), strips or chromatography columns as the solid support. The particles and fusion proteins readily adhere to solid supports. The particles may after purification be disrupted into fusion proteins and the fusion proteins may be dispersed on surfaces as indicated above. These reagents are useful for a variety of diagnostic tests. For example, a test sample suspected of having antibody to the particulate antigen and fluorescent, enzyme or radio-labelled antibody is competitively reacted with the particulate antigen or fusion protein on a solid support and the amount of labelled antibody which binds to the particulate antigen on the solid support. Particulate antigens of this invention are also useful for agglutination reactions with antibodies. Those skilled in the diagnostic arts will recognise a wide variety of application of particulate antigens and fusion proteins of this invention for diagnostic purposes.

Preferred features for each aspect of the invention are as for the first aspect *mutatis mutandis*.

The following examples illustrate the invention, but are not intended to limit the scope in any way. The examples refer to the accompanying drawings, in which;

Figure 1 shows Pepscan analysis of mouse sera. Each plot shows OD_{492} (abscissa) versus peptide number (ordinate) from 1 at the N-terminus to 187 at the truncated C terminus of p1, showing the reactivity of each peptide to antibodies in the test serum. Each test serum is from the pooled sera of five inbred mice immunised with OGS200 VLPs (described below) in the indicated adjuvant.

Figure 1a: RIBI;	1b: SAF-1;	1c: Chemivax

(continued)

1d: normal mouse serum	1e: Alum	1f: unadjuvanted

Figure 2 shows data from pre-absorption experiments used to determine epitope surface accessability in three separate rats. The upper plots show Pepscan activities in sera from rats immunised with OGS200 VLPs in alum. The lower plots show the same sera after preincubation with MA5260 VLPs at 4°C overnight and Pepscan analysis. The loss of reactivity with the peptides is due to sequestration of antibodies by epitopes at the surface of the native VLP. The loss of reactivity is specific for certain epitopes.

Figure 3 shows a summary of surface accessibility of epitopes of pl. The sera used for this summary are from two rabbits immunised with MA5260 VLPs in Freunds and three rats immunised with OGS200 VLPs in alum. The discontinuous bar represents those areas of the p1 protein recognised in the pepscan analysis by antibodies in these sera.

Figure 4 shows the location of the insertion sites A, B, C1 and C2 within the regions A, B and C of p1, as defined by the reactive peptides in the Pepscan analysis. The numbers at the end of each sequence are the p1 amino acid coordinates.

Figure 5 shows plasmid pOGS440.

Example 1 Identification of Epitopes in p1

The PEPSCAN™ kit (CRB, Cambridge) which was prepared for Ty comprises 10-mer peptides overlapping by 8 residues corresponding to the entire length of the wild type p1 protein of Ty1. 187 peptides cover the truncated p1 protein. Each well of a microtitre plate was coated with a peptide and the anti-Ty test serum overlaid. Antibody binding to epitope peptides was detected by a secondary antibody conjugate and a colorimetric reaction.

Sera from five species (human, macaque, rabbit, rat and mouse) were obtained following immunisation with a variety of VLPs (OGS200: p1-HIVp24 (disclosed in WO-A-8803562), MA5620: p1 alone (disclosed in WO-A-8803563), OGS561 : p1-IIIB:MN:RF V3 loops and OGS530.

OGS 561 is a derivative of pOGS 40, which is disclosed in copending patent application PCT/GB92/01545. At the 3'end of TyA gene are three consecutive V3 loops, in order HXBII, MN, RF. These comprise the amino acid sequences

SNCTRPNNNTRKRIRIQRGPGRAFVTIGKIGMMRQAHCNISG (SEQ ID 1)

SNCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNISG (SEQ ID 2)

SNCTRPNNNTRKSITKGPGRVIYATGQIIGDIRKAHCNLSGS (SEQ ID 3)

which are linked by Bam H1 sites which encode two redundant amino acids glycine and serine. The corresponding nucleotide sequences could readily be determined by persons skilled in the art.

pOGS 530 (and pOGS 531 discussed below) are derivatives of pOGS 40, which is disclosed in copending patent application PCT/GB92/01545. These have an oligonucleotide insertion in the Bam H1 site which encodes the MN (Example 10) or HXBII V3 loop respectively. The immunisations were carried out in different adjuvants (alum, RIBI DETOX™, CHEMIVAX™, SAF-1 or Freund's complete). Sera were analysed by PEPSCAN™. Figure 1 shows a typical raw data set from pooled groups of five mice immunised with OGS200 VLPs in different adjuvants. A summary of peptides recognised by all the sera tested is collated in Table 1. The number of epitopes is, to some extent, adjuvant dependent. A summary of the mouse data from Figure 1 is shown in Table 2 to illustrate this dependence by comparing no adjuvant, alum, CHEMIVAX, RIBI and SAF-1. The use of any of the four adjuvants elicits antibodies to more epitopes than no adjuvant. SAF-1 causes antibodies to be raised to more epitopes (8) than RIBI (5), CHEMIVAX (4) and alum (4). A similar effect has been seen in rabbits. Sera from rabbits immunised with OGS200 VLPs in alum recognised a total of 8 p1 epitopes, whereas with SAF-1 12 epitopes were recognised (Table 1). Freund's appears to be the most powerful adjuvant. Nineteen p1 epitopes were recognised by sera from rabbits immunised with OGS5620 VLPs in Freund's (Table 1)

The choice of epitopes for engineering is extensive; however three "consensus" epitopes emerge from the data. These are contained within peptides 11-17, 28-33 and a larger region covered by peptides 47-68. These correspond to amino acid residues 21-42, 55-74 and 93-142 of the p1 protein and have been named A, B and C, respectively. They are recognised by the overwhelming majority of sera, irrespective of the immunising VLP and the adjuvanting regime.

Table 1 shows the reactivity of animal sera to p1 peptides in the Pepscan analysis. Each cell in the table shows the number of responders over background, blanks indicate no response. Of the 16 human clinical trial sera tested, only one had a sufficiently high anti-Ty titre to give reliable reactivities in the Pepscan analysis. Eliminating the remaining 15 non-responders, the maximum possible score in the total column is 33. The three 'consensus' epitopes, A, B and C correspond to the peptides 11-17, 28-33 and 47-68, respectively.

Table 2 shows the serum reactivities of mice immunised with OGS200 VLPs in a variety of adjuvants. All immunisations were intramuscular. The shaded rows correspond to the three "consensus" epitopes, A, B and C at peptides 11-17, 28-33 and 47-68.

Table 1

Peptide Number	Human OGS200 Alum	Macaque OGS200 Alum	Rat OGS200 Alum	Rat OGS561 Alum	Mouse OGS200 Various	Rabbit OGS200 Alum	Rabbit OGS200 SAF-1	Rabbit MA5620 Freunds	Rat MA5620 Alum	Total
1-6		4/4		2/5	1/5	4/5	4/5	2/2		17
11-19	1/16	4/4	5/5	5/5	5/5	5/5	5/5	2/2	1/1	38
21-23					3/5	1/5				4
24-27		3/4		2/5		2/5	1/5	2/2		10
28-33	1/16	3/4	5/5	5/5	5/5	4/5	5/5	2/2	1/1	31
37-42	1/16	3/4			2/5			1/2		7
47-68	1/16	4/4	5/5	5/5	5/5	1/5	5/5	2/2	1/1	33
72-77		2/4		2/5		3/5	4/5	1/2		12
81-85	1/16	3/4	1/5	2/5	1/5		1/5	1/2		10
88-92	1/16			3/5	2/5			1/2		7
101-102		1/4						2/2		3
112-118		4/4		1/5			4/5	2/2		11
128-134		4/4	3/5				4/5	2/2	1/1	14
139-140	1/16	4/4	1/5	1/5				2/2		9
144-146			2/5	4/5			1/5			7
150-151			3/5					2/2		5
157-161		1/4		1/5			1/5	1/2	1/1	5
169-171		1/4	1/5					2/2		4
175-182		3/4	5/5	2/5		1/5	4/5	2/2	1/1	18
185-187						1/5		1/2		2

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Table 2

Peptide Number	ADJUVANT				
	None	Alum	Ribi	Chemivax	SAF-1
1-6	-	-	-	-	+
11-17	+	+	+	+	+
21-23	-	-	+	+	+
28-33	+	+	+	+	+
37-42	-	+	-	-	+
47-68	+	+	+	+	+
81-85	-	-	-	-	+
88-92	-	-	+	-	+

Example 2 Identification of Surface Epitopes of p1

PEPSCAN™ analysis will identify any well defined linear epitope of p1. Since the analysis is based on recognition of short linear peptides, conformational or non-contiguous epitopic determinants are unlikely to be detected. In addition, PEPSCAN™ data do not distinguish between surface (*ie* accessible to the antibody) or buried epitopes of the native VLP.

Serum preabsorption studies were used to determine which regions of p1, and in particular which of the three epitopes identified above, are surface accessible. Sera from three rats immunised with OGS200 VLPs in alum and from two rabbits immunised with MA5620 VLPs in Freund's were incubated with native purified MA5620 VLPs. These sera were then analysed by PEPSCAN™. Antibodies to surface accessible epitopes bind to the surface of the native VLP and are therefore unavailable to bind to the PEPSCAN™ peptides. Where an epitope is surface accessible, a loss of previously observed reactivity with that epitope indicates that it is a surface feature. The preabsorption experiments were controlled for proteolysis of the native VLP by serum proteases by analysing the particles post-absorption by western blot.

Figure 2 shows the PEPSCAN™ data from three rat sera before and after preabsorption. These data show that epitopes in the N terminal half of p1 are mostly surface accessible whereas those in the C terminal half of the protein are mainly inaccessible. The three major linear epitopes, A, B and C identified above, all showed surface accessibility. A diagrammatic summary of preabsorption experiment data is shown in Figure 3 which illustrates the accessibility of linear epitopes of p1. The gaps are due to regions of the protein not recognised by antibodies in any of the sera tested. This analysis demonstrates that surface accessibility, where information is available, is essentially limited to the N terminal half of p1.

Example 3 Choice of p1 Epitopes for Engineering

The three consensus epitopes identified satisfy several criteria for selection as targets for insertion of antigen: they are recognised by sera from all species tested irrespective of the VLP type used as an immunogen and the adjuvanting regime; and all are surface exposed.

Four insertion points within the p1 protein were chosen, one each in A and B, and two in C. These lie between amino acids 30-31, 67-68, 113-114 and 132-133 and are referred to as A, B, C1 and C2 respectively (see Figure 4). Although these four sites were chosen for evaluation, other positions within the defined regions A, B and C may be

equally appropriate as insertion sites.

Example 4 Manipulation of the TYA(d) Gene

The TyA (d) gene was manipulated to introduce a unique *Nhe* I restriction site at insetion points A, B, C1 or C2 to allow insertion of foreign DNA sequences. Four versions were thus constructed, one for each of the four insertion points selected. The vectors containing this modification were prepared as follows. A *BglII/BamH1* restriction fragment containing the coding sequence of the TyA(d) gene was excised from pOGS 226 and inserted into the vector pSP46 also digested with *BglII/BamH1,* to give pOGS460 (pSP46 is a derivative of pSP64 in which the *HindIII* site in the polylinker has been converted to a *BglII* site).

pOGS 460 was then digested with *NheI* (restriction site present within pSP46) and *PstI* (restriction site present within TyA gene) to release a 1117bp fragment. This was then inserted into M13 mp18 digested with XbaI and PstI. Using site directed mutagenesis, an *Nhe* I restriction site was then introduced at the insertion points A, B, C1 or C2 (ie between TyA nucleotides 90-91, 201-202, 339-340 and 396-397 respectively)

The *NheI* site was used for insertion of double stranded (ds) oligonucleotides encoding each of three size variants of the MN isolate V3 loop.

The mutagenised TyA(d) sequences were removed from M13 as *BglII/SpII* fragments and ligated into the vector backbone of *BglII/SpII* digested pOGS440. The *SpII* restriction site in the TyA (d) gene is 5' to the *Pst I* site.

These manipulations yielded the following plasmid constructions:

pOGS810 is the pOGS440 equivalent with the *NheI* site at position A
pOGS811 " B
pOGS812 " C_1
pOGS813 " C_2

pOGS440 was constructed as follows. pKV560 is described by Chambers *et al.,* (1989) Mol. Cell. Bio. 9 5516-5524. pKV572 is identical to pKV560 with the exception that the interferon sequences are removed leaving a *BglII* cloning site. pKV572 contains the minimal assay promoter with a 5' *BamH1* cloning site for upstream activating sequences, and is the starting point for pJC87.

A 1kb EcoR1-Xho1 fragment from pUG4IS containing the GAL-10 promoter sequence was purified. This was further digested with *Dde1* and a 510 base pair fragment isolated. The 5' protruding ends of this fragment were filled-in with the Klenow fragment of DNA polymerase and BglII oligonucleotide linkers added.

The fragment was then digested with *Sau3A* and a 360 base pair fragment purified. This fragment was ligated into BamH1 digested, phosphate treated pKV572. The ligated products were transformed into HW87 and the resultant plasmids screened for the orientation of the insert by DNA sequencing. A clone which had the 360 base pair GAL1-10 *Dde1-Sau3A* fragment in the GAL1 orientation was selected and called pJC78.

pOGS440 is shown in Figure 5; it was constructed by inserting the BglII/SalI fragment from pOGS226 (a derivative of pMA5620 described in WO-A-8803563 which has an additional BglII site inserted adjacent to the N-terminus of p1) into BglII/SalI at pJC78.

Example 5 Particle Formation by Insertion Site Mutants of p1

Insertion of an *NheI* restriction site into the TyA(d) gene as described in Example 4 resulted in the introduction of two additional amino acids (Alanine and Serine) into the pI protein. It was necessary to confirm that this change did not interfere with particle formation for any of the chosen insertion sites (A, B, C1 or C2).

Plasmids pOGS810, pOGS811, pOGS812 and pOGS813 were transformed into *S. cerevisiae* strain MC2, although any available strain could be used. The transformed cells were cultured, harvested and the VLPs isolated by fractionation on sucrose gradients as follows.

Yeast cells were grown selectively at 30°C to a density of $8x10^6$ cells/ml. The cells were then collected by low speed centrifugation, washed once in ice-cold water and resuspended in TEN buffer (10mM Tris, pH 7.4, 2mM EDTA, 140mM NaCl) at 1ml per 1 litre of cells. The cells were disrupted by vortexing with glass beads (40-mesh, BDH), at 4°C until >70% were broken. The beads were pelleted by low speed centrifugation (2,000g), then the supernatant was collected, and the debris removed by centrifugation at 13, 000g for 20 minutes.

The clarified supernatant was transferrred to a SW28 tube and underlayered with 3ml of 60% w/v sucrose solution in TEN. Tubes were then centrifuged at 28K rpm for 90minutes to band the VLPs at the sucrose interface.

VLPs were recovered and dialysed against TEN to remove the sucrose, then purified further by banding on a pre-formed linear (10-60%) sucrose gradient (SW41 tubes centrifuged at 25krpm for 6 hours). The VLPs were recovered, dialysed and concentrated.

All four constructions expressed particulate p1 protein at levels comparable to the positive control for the experiment, pOGS440, demonstrating that addition of the two residues at the insertion points does not adversely affect particle formation.

Example 6 Insertion of Antigen $(GPGRAF)_3$

Complementary pairs of DNA oligonucleotides were synthesised encoding the central six residues (GPGRAF) of the gp120 V3 loop from the MN isolate. These are

$$(5'CTAGTGGTCCAGGTAGAGCTTTCT3')_3 \text{ (SEQ ID 4)}$$

The ends of the annealed double stranded oligonucleotide are compatible with *Nhe*I cut ends for ligation into the unique *Nhe*I sites within pOGS810-813. Transformants were initially screened for the absence of the *Nhe*I site which is abolished by oligonucleotide insertion before DNA sequencing for verification. Three tandemly repeated copies were inserted at position B in the TYA(d) gene of pOGS811, to generate pOGS814. The coding consequence of this is as follows:

P E N P A S **G P G R A F S S G P G R A F S S G P G R A F** S S H H A S P
(SEQ ID 5)

The residues in bold are the inserted amino acids flanked by the wild type p1 residues. The A S and S S motifs are encoded by the *Nhe*I cohesive ends of the oligonucleotide.

Construction of the $(GPGRAF)_3$ insert has provided information on the size of insert that can be tolerated at position B. Since this 26 residue insertion allows particle formation, insertion of the 20 and 40 residue V3 loop size variants should be tolerated at that position. It also supports the notion that the original Ty epitope is in the form of a surface loop which can be extended without interfering with the normal fold of the p1 monomer.

Example 7 Insertion of Antigen: GPGRAF

Oligonucleotides encoding for GPGRAF with *Nhe*I compatible ends were synthesised as described above.

Once annealed they were ligated into each of the four insertion sites. Once the oligonucleotide was inserted, the *Nhe*I site was abolished. Resulting transformants were therefore screened for loss of the *Nhe*I site. The orientation of the insert was verified by DNA sequencing. The resulting constructions are numbered as follows:

pOGS815:	pOGS810 with GPGRAF at position	A
pOGS816:	pOGS811	B
pOGS817:	pOGS812	C1
pOGS818:	pOGS813	C2

The total inserted sequence is as follows:

A S G P G R A F S S (SEQ ID 6)

The AS and SS residues flanking the N and C termini of the inserted antigen respectively are encoded by the altered *Nhe*I sites at each end of the inserted oligonucleotide.

S. cerevisiae strain MC2 yeast cells were transformed with each plasmid.

Example 8 Insertion of Antigen: Half V3 loop

Complementary pairs of DNA oligonucleotides

5'CTAGTAAAAGAAAGAGAATTCATATTGGTCCAGGTAGAGCTTTCTATAC TACCAAAAACATTATCG3' (SEQ ID 7)

were synthesised that encode the following sequence:

A S K R K R I H I G P G R A F Y T T K N I I A S (SEQ ID 8)

The flanking AS residues are those encoded by the *Nhe*I compatible oligonucleotide ends. The annealed oligonucleotide possessed an *Eco*RI restriction site. Once ligated into the vector the 5' *Nhe*I site was abolished while the 3' *Nhe*I site was recreated. The remaining 3' *Nhe*I site enables further antigens to be added if desired. Transformants were screened by *Eco*RI restriction digestion and the orientation of insertion was determined by DNA sequencing. The resulting constructions are numbered as follows:

pOGS819:	pOGS810 with the half V3 loop at position	A
pOGS820:	pOGS811	B
pOGS821:	pOGS812	C1
pOGS822:	pOGS813	C2

Example 9 Insertion of Antigen: Whole V3 loop

Two pairs of complementary pairs of DNA oligonucleotides

5'CTAGTATTAATTGCACCCGTCCTAACTACAATAAAAGAAAGAGAATTCA TATTGGTCCAGGT3' (SEQ ID 9) and

5'AGAGCTTTCTATACTACCAAAAACATTATCGGTACTATTAGACAAGCTC ACTGTAATATCG3' (SEQ ID 10)

were synthesised that together encode the whole V3 loop sequence as follows:

A S I N C^S T R P N Y N K R K R I H I G P G R A F Y T T K N I I G T I R Q A H C^S N I A S. (SEQ ID 11)

The flanking AS residues were encoded by the *Nhe*I compatible ends and C^S signifies the cysteine residues thought to close the loop at its base by a disulphide bond. The whole insert was constructed in two parts which were ligated together before ligation into the appropriate vectors. As with the half loop oligonucleotides, the 5' *Nhe*I site is abolished on insertion and the 3' *Nhe*I site is recreated. The inserted sequence also carries an *Eco*RI restriction site to aid screening. The resulting transformants were screened for the presence and orientation of the DNA fragment by restriction enzyme digestion. The three ligation junctions, at each end and in the middle of the insert, were verified by DNA sequencing. The constructions were numbered as follows:

pOGS823:	pOGS810 with whole V3 loop at position	A
pOGS824:	pOGS811	B
pOGS825:	pOGS812	C1

(continued)

pOGS826:	pOGS813	C2

Example 10 Characterisation of pOGS814 VLPs: $(GPGRAF)_3$ at position B

Purified pOGS814 DNA was transformed into *S. cerevisiae* strain MC2, although any available strain could be used. Cells were harvested, hand bead-beaten and the cell homogenate clarified by centrifugation at 9K for 20 minutes. 1.5ml of this material was then applied to sucrose gradients (15 to 45% with a 60% cushion) and centrifuged at 40 Krpm for 1.5 hours. The gradients were fractionated and examined by SDS-PAGE. The OGS814 protein sedimented with the characteristics of a VLP in a well defined zone half way down the gradient, well resolved from monomeric protein solutes.

Example 11 Immunoreactivity of OGS814 VLPs

Fractions from the gradients described in Example 10 were analysed by western blotting with three antibodies: an anti-Ty polyclonal, DuPont gp120 MAb 9305 which reacts with the V3 loop tip sequence -RIQRGPGRAFVTIGK-, and Dupont gp120 monoclonal 9284, which reacts with the left-hand side of the V3 loop-NNNTRKSIRIQR-. As expected, the OGS814 VLPs reacted with the Ty polyclonal and 9305 MAb, but not with 9284 MAb. The controls were MA5620 VLPs and OGS531 VLPs (whole V3 loop from isolate HXB2 at the C terminus). All the controls had the predicted reactivities. The western blot data are summarised in Table 3.

Table 3.

Western blot immunoreactivity data from MA5620, OGS814, and OGS531 VLPs with anti-Ty, 9305 and 9284 antibodies.					
VLP	Added Antigen	Antigen position	Antibody		
			anti-Ty	9305	9284
MA5620	-	-	+	-	-
OGS814	$(GPGRAF)_3$	B	+	+	-
OGS531	V3 loop	C terminus	+	+	+

Example 12 Surface Exposure of the Antigen in OGS814 VLPs

The p1 epitope at position B was shown to be surface-exposed in native whole VLPs by its ability to bind its cognate antibody which could then be removed from solution by cosedimentation with the VLP during centrifugation. A similar approach was used to demonstrate that the GPGRAF component of OGS814 at position B is also surface exposed. In this case the cognate antibody was the MAb 9305, shown to recognise OGS814 VLPs. The experiment involved incubation of the VLPs with the MAb, pelleting the VLPs by centrifugation and measuring the amount of unbound MAb left in the supernatant using a V3 peptide ELISA.

In an ELISA for detecting MAbs binding to the 40 amino acid HXB2 gp120 V3 loop peptide, VLPs at 100 and 500 µg/ml or peptide at 200 µg/ml were incubated with 9305 or 9284 MAb at a dilution of 1/100 from the stock. Controls for binding in solution were MA5620 VLPs (negative) and the V3 peptide (positive). The mixtures were centrifuged at 75 Krpm for 15 minutes and the supernatants assayed for residual MAb by ELISA. In summary:

1) the MAbs alone were not removed from solution by centrifugation
2) the negative control MA5620 VLPs bound no antibody, which remained in the supernatant
3) the positive control peptide removed all antibody reactivity from the supernatant, *ie* no unbound antibody remained
4) OGS814 VLPs bound 9305, but not 9284 antibodies, indicating that the GPGRAF motif in these VLPs is surface accessible

Example 13 Immunogenicity of OGS814 VLPs

Rats were immunised with purified OGS814 VLPs. Rats were primed at week 0, boosted at weeks 6 and 12, and final bleeds were taken at week 14. Intermediate test bleeds were taken at weeks 6, 8 and 12. Two doses of 50 and

250 µg per immunisation, in the presence and absence of adjuvant are given to four groups of five rats as follows:

Group 1 50 µg - adjuvant/animal
Group 2 50 µg + adjuvant/animal
Group 3 250 µg - adjuvant/animal
Group 4 250 µg + adjuvant/animal

Example 14 Immunogenicity of OGS822 VLPs

OGS822 VLPs were chosen to examine the improved immunogenicity resulting from the insertion of the half V3 loop within the Ty p1 protein. Rats were immunised intramuscularly with 250µg purified OGS822 VLPs in aluminium hydroxide adjuvant. Rats were primed at week 0, boosted at weeks 6 and 12, and final bleeds were taken at week 14. Sera were tested for anti-V3 antibody responses both by ELISA and neutralisation assays, the results of which are shown in Table 4.

Table 4

Serum antibody and neutralising antibody titres of rats immunised with OGS 822 VLPs. The ELISA data (shown as units/ml) are arbitrary values based on a standard curve produced with a rat anti-MN peptide antiserum. Neutralising antibody titres are expressed as the dilution of serum that resulted in 90% inhibition of syncytia formation in a standard assay.

	4 weeks post-prime		2 weeks post boost	
ANIMAL	ELISA U/ml	neutralising	ELISA U/ml	neutralising
1	21.4	320-640	28.4	1280
2	23.1	640	44.4	1280-2560
3	13.1	256	11.7	320-640
4	8.0	256	8.2	640
5	-	16	4.2	256

In the same assays, a pool of antisera from rats immunised with OGS259 VLPs (1/2 V3 loop at the C-terminus) generated an ELISA value of 2.27 U/ml and a neutralisation titre of 1:8. Insertion of antigen at an internal site (in this case C2) thus resulted in a dramatic improvement in immunogenicity.

Example 15 Insertion of Antigen: Influenza nucleoprotein CTL epitope

Complementary pairs of DNA oligonucleotides were synthesised that encode the following sequence:

ASRS **TYQRTRALV** GSAS (SEQ ID 12)

This contains an influenza nucleoprotein CTL epitope (shown in bold). The flanking ASRS and GSAS amino acids are encoded by restriction enzyme sites. This sequence was inserted into the p1 protein at each of the four sites A, B, C_1 and C_2.

Claims

1. A non-natural particle-forming protein comprising a self-assembling particle-forming first amino acid sequence substantially homologous with a yeast retrotransposon Ty p1 protein and a second amino acid sequence, wherein the second sequence is antigenic and is incorporated within an epitope of the first amino acid sequence, which epitope, on particles formed from the first amino-acid sequence alone, is surface-exposed.

2. A particle-forming protein as claimed in claim 1 wherein the second amino acid sequence is inserted into the surface-exposed epitope.

3. A particle-forming protein as claimed in claim 1 wherein the second amino acid sequence is substituted in place of native amino acids normally present in the surface-exposed epitope.

4. A particle-forming protein as claimed in any of claims 1 to 3 wherein native amino acids normally present in the surface epitope are deleted.

5. A particle-forming protein as claimed in any of claims 1 to 4 wherein the surface-exposed epitope is present in the N-terminal half of the first amino acid sequence.

6. A particle-forming protein as claimed in claim 5 wherein the first amino acid sequence p1 protein of the retrotransposon Ty has been truncated at the C-terminal.

7. A particle-forming protein as claimed in claim 6 wherein the surface-exposed epitope is located between amino acids 21-42.

8. A particle-forming protein as claimed in claim 6 wherein the surface-exposed epitope is located between amino acids 55-74.

9. A particle-forming protein as claimed in claim 6 wherein the surface-exposed epitope is located between amino acids 93-142.

10. A particle-forming protein as claimed in claim 7 wherein the second amino acid sequence is inserted between amino acids 30-31,

11. A particle-forming protein as claimed in claim 8 wherein the second amino acid sequence is inserted between amino acids 67-68

12. A particle-forming protein as claimed in claim 9 wherein the second amino acid sequence is inserted between amino acids 113-114

13. A particle-forming protein as claimed in claim 9 wherein the second amino acid sequnece is inserted between amino acids 132-133

14. A particle-forming protein as claimed in any one of claims 1 to 13 wherein the antigenic second amino acid sequence or sequences correspond to a sequence derived from or associated with an aetiological agent or a tumour.

15. A particle-forming protein as claimed in claim 14 wherein the aetiological agent is a microorganism such as a virus, bacterium, fungus or parasite.

16. A particle-forming protein as claimed in claim 15 wherein the virus is a retrovirus, for example HIV-1, HIV-2, HTLV-I, HTLV-II, HTLV-III, SIV, BIV, ELAV, CIAV, murine leukaemia virus, Moloney murine leukaemia virus, and feline leukaemia virus; an orthomyxovirus, for example influenza A or B; a paramyxovirus, for example parainfluenza virus, mumps, measles, RSV and Sendai virus; a papovavirus, for example HPV; an arenavirus, for example LCMV of humans or mice; a hepadnavirus, for example Hepatitis B virus; or a herpes virus, for example HSV, VZV, CMV, or EBV.

17. A particle-forming protein as claimed in claim 14 wherein the tumour-associated or derived antigen is a proteinaceous human tumour antigen, for example a melanoma-associated antigen, or an epithelial-tumour associated antigen, for example from breast or colon carcinoma.

18. A particle-forming protein as claimed in claim 14 wherein the antigenic sequence is derived from a bacterium, for example of genus *Neisseria, Bordetella, Listeria, Mycobacteria* or *Leishmania,* from a parasite, for example from the genus *Plasmodium,* or from a fungus, for example from the genus *Candida, Aspergillus, Cryptococcus, Histoplasma* or *Blastomyces.*

19. A particle-forming protein as claimed in any of claims 1 to 18 wherein the antigenic sequence is between 6 and 60 amino acids in length.

20. A particle-forming protein as claimed in claim 14 wherein the antigenic sequence is an epitope from:

1) HIV (particularly HIV-1) gp120,
2) HIV (particularly HIV-1) p24,
3) Influenza virus nucleoprotein and haemagglutinin,
4) LCMV nucleoprotein,
5) HPV L1, L2, E4, E6 and E7 proteins,
6) p97 melanoma associated antigen,
7) GA 733-2 epithelial tumour-associated antigen,
8) MUC-1 epithelial tumour-associated antigen,
9) Mycobacterium p6,
10) Malaria CSP or RESA antigens,
11) VZV gpI, gpII or gpIII

21. A particle-forming protein as claimed in claim 20 wherein the epitope is the V3 loop or GPGR loop of the envelope glycoprotein gp120 of a lentivirus.

22. A particle-forming protein as claimed in any of the preceding claims wherein two or more of the surface exposed epitopes have an antigenic amino acid sequence incorporated therein.

23. A particle-forming protein as claimed in claim 22 wherein the antigenic amino acid sequence incorporated within one of the surface-exposed epitopes is different from the antigenic amino acid sequence incorporated within another of the surface-exposed epitopes.

24. A particle-forming protein as claimed in any of the preceding claims wherein more than one antigenic amino acid sequence is incorporated within any single surface exposed epitope.

25. A particle-forming protein as claimed in claim 24 wherein the antigenic amino acid sequences incorporated in any single surface exposed epitope are not all identical.

26. A protein as claimed in claim 24 or claim 17 wherein the antigenic amino acid sequences are incorporated in tandem within the surface exposed epitope.

27. A protein as claimed in claims 23, 25 or 26 where the different second amino acid sequences are derived from different epitopes of the same antigen.

28. A particle comprising a plurality of homologous proteins as claimed in any of claims 1 to 27.

29. A particle comprising a plurality of heterologous proteins as claimed in any of claims 1 to 27.

30. Nucleic acid coding for a fusion protein as claimed in any one of claims 1 to 27.

31. A vector including nucleic acid as as claimed in claim 30.

32. A host cell carrying a vector as claimed in claim 31.

33. A host cell as claimed in claim 32 where the host cell is *E. coli*

34. A host cell as claimed in claim 32 where the host cell is a yeast cell for example *Saccharomyces cerevisiae* or *Pichia pastoris*

35. Host cells as claimed in claim 32 where the host cell is an insect cell for example *Spodoptera frugiperda*.

36. Antibodies raised or directed against particulate antigens as claimed in any of claims 1 to 29.

37. The use of hybrid proteins and/or particles as claimed in any one of claims 1 to 29 in the preparation of an immunotherapeutic or prophylactic vaccine.

38. The use of particulate antigens as claimed in claims 1 to 29 as a diagnostic agent.

39. A pharmaceutical or veterinary composition comprising a protein as claimed in any one of claims 1 to 29 together with a pharmaceutically and/or veterinarily acceptable carrier.

Patentansprüche

1. Nicht-natürliches, partikelbildendes Protein, umfassend eine selbstanordnende, partikelbildende erste Aminosäuresequenz, die im wesentlichen homolog mit einem Hefe-Retrotransposon Ty p1-Protein ist, und eine zweite Aminosäuresequenz, worin die zweite Aminosäuresequenz antigen ist und innerhalb eines Epitops der ersten Aminosäuresequenz eingebaut ist, wobei das Epitop auf der Oberfläche der allein aus der ersten Aminosäuresequenz gebildeten Partikeln exponiert ist.

2. Partikelbildendes Protein nach Anspruch 1, worin die zweite Aminosäuresequenz in das oberflächenexponierte Epitop eingefügt ist.

3. Partikelbildendes Protein nach Anspruch 1, worin die zweite Aminosäuresequenz anstelle der normalerweise im oberflächenexponierten Epitop vorhandenen nativen Aminosäuren substituiert ist.

4. Partikelbildendes Protein nach einem der Ansprüche 1 bis 3, worin die normalerweise in dem Oberflächenepitop vorhandenen nativen Aminosäuren deletiert sind.

5. Partikelbildendes Protein nach einem der Ansprüche 1 bis 4, worin das oberflächenexponierte Epitop in der N-terminalen Hälfte der ersten Aminosäuresequenz vorliegt.

6. Partikelbildendes Protein nach Anspruch 5, worin das erste Aminosäuresequenz-p1-Protein des Retrotransposons Ty am C-Terminus verkürzt wurde.

7. Partikelbildendes Protein nach Anspruch 6, worin das oberflächenexponierte Epitop zwischen den Aminosäuren 21-42 lokalisiert ist.

8. Partikelbildendes Protein nach Anspruch 6, worin das oberflächenexponierte Epitop zwischen den Aminosäuren 55-74 lokalisiert ist.

9. Partikelbildendes Protein nach Anspruch 6, worin das oberflächenexponierte Epitop zwischen den Aminosäuren 93-142 lokalisiert ist.

10. Partikelbildendes Protein nach Anspruch 7, worin die zweite Aminosäuresequenz zwischen den Aminosäuren 30-31 eingefügt ist.

11. Partikelbildendes Protein nach Anspruch 8, worin die zweite Aminosäuresequenz zwischen den Aminosäuren 67-68 eingefügt ist.

12. Partikelbildendes Protein nach Anspruch 9, worin die zweite Aminosäuresequenz zwischen den Aminosäuren 113-114 eingefügt ist.

13. Partikelbildendes Protein nach Anspruch 9, worin die zweite Aminosäuresequenz zwischen den Aminosäuren 132-133 eingefügt ist.

14. Partikelbildendes Protein nach einem der Ansprüche 1 bis 13, worin die antigene zweite Aminosäuresequenz oder -sequenzen einer Sequenz entsprechen, die von einem auslösenden Agens oder einem Tumor abgeleitet ist, oder damit assoziiert ist.

15. Partikelbildendes Protein nach Anspruch 14, worin das auslösende Agens ein Mikroorganismus ist, wie ein Virus, Bakterium, Pilz oder Parasit.

16. Partikelbildendes Protein nach Anspruch 15, worin das Virus ein Retrovirus ist, z.B. HIV-1, HIV-2, HTLV-I, HTLV-

II, HTLV-III, SIV, BIV, ELAV, CIAV, Murin-Leukämie-Virus, Moloney-Murin-Leukämie-Virus und Felin-Leukämie-Virus; ein Orthomyxovirus, z.B. Influenza A oder B; ein Paramyxovirus, z.B. Parainfluenza-Virus, Mumps, Masern, RSV und Sendai-Virus; ein Papovavirus, z.B. HPV; ein Arenavirus, z.B. LCMV von Menschen oder Mäusen; ein Hepadnavirus, z.B. Hepatitis B-Virus; oder ein Herpes-Virus, z.B. HSV, VZV, CMV oder EBV.

17. Partikelbildendes Protein nach Anspruch 14, worin das Tumor-assoziierte oder abgeleitete Antigen ein proteinartiges humanes Tumorantigen ist, z.B. ein Melanomassoziiertes Antigen oder ein Epithelial-Tumor-assoziiertes Antigen, z.B. aus einem Brust- oder Colonkarzinom.

18. Partikelbildendes Protein nach Anspruch 14, worin die antigene Sequenz von einem Bakterium abgeleitet ist; z. B. von der Gattung *Neisseria, Bordetella, Listeria, Mycobacteria* oder *Leishmania,* von einem Parasiten, z.B. von der Gattung *Plasmodium,* oder von einem Pilz, z.B. von der Gattung *Candida, Aspergillus, Cryptococcus, Histoplasma* oder *Blastomyces.*

19. Partikelbildendes Protein nach einem der Ansprüche 1 bis 18, worin die antigene Sequenz zwischen 6 und 60 Aminosäuren lang ist.

20. Partikelbildendes Protein nach Anspruch 14, worin die antigene Sequenz ein Epitop ist von:

1) HIV (insbesondere HIV-1)gp120,
2) HIV (insbesondere HIV-1)p24,
3) Influenza-Virus Nukleoprotein und Hämagglutinin,
4) LCMV-Nukleoprotein,
5) HPV L1-, L2-, E4-, E6- und E7-Proteine,
6) p97-Melanom-assoziiertes Antigen,
7) GA 733-2-Epithelial-Tumor-assoziiertes Antigen,
8) MUC-1-Epithelial-Tumor-assoziiertes Antigen,
9) Mycobacterium p6,
10) Malaria CSP- oder RESA-Antigenen,
11) VZV gpI, gpII oder gpIII

21. Partikelbildendes Protein nach Anspruch 20, worin das Epitop die V3-Schlaufe oder GPGR-Schlaufe des Glyproteins gp120 eines Lentivirus ist.

22. Partikelbildendes Protein nach einem der vorhergehenden Ansprüche, worin zwei oder mehr oberflächenexponierte Epitope eine darin eingebaute antigene Aminosäuresequenz haben.

23. Partikelbildendes Protein nach Anspruch 22, worin die antigene Aminosäuresequenz, die in einem der oberflächenexponierten Epitope eingebaut ist, von der in anderen oberflächenexponierten Epitopen eingebauten antigenen Aminosäuresequenz unterschiedlich ist.

24. Partikelbildendes Protein nach einem der vorhergehenden Ansprüche, worin mehr als eine antigene Aminosäuresequenz in einem einzelnen oberflächenexponierten Epitop eingebaut ist.

25. Partikelbildendes Protein nach Anspruch 24, worin die antigenen Aminosäuresequenzen, die in irgendeinem einzelnen exponierten Epitop eingebaut sind, nicht alle identisch sind.

26. Protein nach Anspruch 24 oder Anspruch 17, worin die antigenen Aminosäuresequenzen in Tandem-Anordnung in dem oberflächenexponierten Epitop eingebaut sind.

27. Protein nach Ansprüchen 23, 25 oder 26, worin die verschiedenen zweiten Aminosäuresequenzen von verschiedenen Epitopen desselben Antigens abgeleitet sind.

28. Partikel, umfassend eine Vielzahl von homologen Proteinen nach einem der Ansprüche 1 bis 27.

29. Partikel, umfassend eine Vielzahl von heterologen Proteinen nach einem der Ansprüche 1 bis 27.

30. Nukleinsäure, kodierend für ein Fusionsprotein nach einem der Ansprüche 1 bis 27.

31. Vektor, umfassend die Nukleinsäure nach Anspruch 30.

32. Wirtszelle, die einen Vektor nach Anspruch 31 trägt.

33. Wirtszelle nach Anspruch 22, worin die Wirtszelle *E. coli* ist.

34. Wirtszelle nach Anspruch 32, worin die Wirtszelle eine Hefezelle, z.B. *Saccharomyces cerevisiae* oder *Pichia pastoris* ist.

35. Wirtszellen nach Anspruch 32, worin die Wirtszelle eine Insektenzelle, z.B. *Spodoptera frugiperda* ist.

36. Antikörper, gebildet oder gerichtet gegen partikelförmige Antigene nach einem der Ansprüche 1 bis 29.

37. Verwendung von Hybridproteinen und/oder - partikeln nach einem der Ansprüche 1 bis 29 bei der Herstellung eines immuntherapeutischen oder prophylaktischen Impfstoffes.

38. Verwendung von partikelförmigen Antigenen nach einem der Ansprüche 1 bis 29 als diagnostisches Mittel.

39. Pharmazeutische oder veterinärmedizinische Zusammensetzung, umfassend ein Protein nach einem der Ansprüche 1 bis 29 zusammen mit einem pharmazeutisch und/oder veterinärmedizinisch verträglichen Träger.

Revendications

1. Protéine non naturelle formant des particules, comprenant une première séquence d'acides aminés à auto-assemblage, formant des particules, sensiblement homologue à une protéine p1 de rétrotransposon Ty de levure, et une deuxième séquence d'acides aminés, dans laquelle la deuxième séquence est antigénique et est incorporée au sein d'un épitope de la première séquence d'acides aminés, ledit épitope étant, sur les particules formées à partir de la première séquence d'acides aminés seule, exposé en surface.

2. Protéine formant des particules selon la revendication 1, dans laquelle la deuxième séquence d'acides aminés est insérée dans l'épitope exposé en surface.

3. Protéine formant des particules selon la revendication 1, dans laquelle la deuxième séquence d'acides aminés remplace les acides aminés natifs normalement présents dans l'épitope exposé en surface.

4. Protéine formant des particules selon l'une quelconque des revendications 1 à 3, dans laquelle les acides aminés natifs normalement présents dans l'épitope en surface sont délétés.

5. Protéine formant des particules selon l'une quelconque des revendications 1 à 4, dans laquelle l'épitope exposé en surface est présent dans la moitié N-terminale de la première séquence d'acides aminés.

6. Protéine formant des particules selon la revendication 5, dans laquelle la protéine p1 de la première séquence d'acides aminés du rétrotransposon Ty a été tronquée au niveau C-terminal.

7. Protéine formant des particules selon la revendication 6, dans laquelle l'épitope exposé en surface est situé entre les acides aminés 21 et 42.

8. Protéine formant des particules selon la revendication 6, dans laquelle l'épitope exposé en surface est situé entre les acides aminés 55 et 74.

9. Protéine formant des particules selon la revendication 6, dans laquelle l'épitope exposé en surface est situé entre les acides aminés 93 et 142.

10. Protéine formant des particules selon la revendication 7, dans laquelle la deuxième séquence d'acides aminés est insérée entre les acides aminés 30 et 31.

11. Protéine formant des particules selon la revendication 8, dans laquelle la deuxième séquence d'acides aminés

est insérée entre les acides aminés 67 et 68.

12. Protéine formant des particules selon la revendication 9, dans laquelle la deuxième séquence d'acides aminés est insérée entre les acides aminés 113 et 114.

13. Protéine formant des particules selon la revendication 9, dans laquelle la deuxième séquence d'acides aminés est insérée entre les acides aminés 132 et 133.

14. Protéine formant des particules selon l'une quelconque des revendications 1 à 13, dans laquelle la ou les deuxièmes séquences d'acides aminés antigéniques correspondent à une séquence dérivée d'un agent étiologique ou d'une tumeur, ou associée à ces demiers.

15. Protéine formant des particules selon la revendication 14, dans laquelle l'agent étiologique est un microorganisme tel qu'un virus, une bactérie, un champignon ou un parasite.

16. Protéine formant des particules selon la revendication 15, dans laquelle le virus est un rétrovirus, par exemple HIV-1, HIV-2, HTLV-I, HTLV-II, HTLV-III, SIV, BIV, ELAV, CIAV, le virus des leucémies murines, le virus des leucémies murines de Moloney, et le virus des leucémies félines; un orthomyxovirus, par exemple les virus de la grippe de type A ou B; un paramyxovirus, par exemple le virus para-influenza, le virus des oreillons, le virus de la rougeole, RSV et le virus Sendai; un papovavirus, par exemple HPV; un arénavirus, par exemple le LCMV humain ou de souris; un hépadnavirus, par exemple le virus de l'hépatite B; ou un herpèsvirus, par exemple HSV, VZV, CMV ou EBV.

17. Protéine formant des particules selon la revendication 14, dans laquelle l'antigène associé à une tumeur ou dérivé de tumeur est un antigène tumoral protéinique humain, par exemple, un antigène associé à un mélanome, ou un antigène associé à une tumeur épithéliale, par exemple, provenant d'un cancer du sein ou du côlon.

18. Protéine formant des particules selon la revendication 14, dans laquelle la séquence antigénique est dérivée d'une bactérie, par exemple, du genre Neisseria, Bordetella, Listeria, Mycobacteria ou Leishmania, d'un parasite, par exemple, du genre Plasmodium, ou d'un champignon, par exemple, du genre Candida, Aspergillus, Cryptococcus, Histoplasma ou Blastomyces.

19. Protéine formant des particules selon l'une quelconque des revendications 1 à 18, dans laquelle la séquence antigénique a une longueur comprise entre 6 et 60 acides aminés.

20. Protéine formant des particules selon la revendication 14, dans laquelle la séquence antigénique est un épitope provenant:

1) de gp120 de HIV (en particulier HIV-1),
2) de p24 de HIV (en particulier HIV-1),
3) de nucléoprotéine et d'hémagglutinine du virus de la grippe,
4) de nucléoprotéine de LCMV,
5) des protéines L1, L2, E4, E6 et E7 de HPV,
6) de l'antigène associé au mélanome p97,
7) de l'antigène associé à la tumeur épithéliale GA 733-2,
8) de l'antigène associé à la tumeur épithéliale MUC-1,
9) p6 de Mycobacterium,
10) des antigènes CSP ou RESA de paludisme,
11) de gpI, gpII ou gpIII de VZV.

21. Protéine formant des particules selon la revendication 20, dans laquelle l'épitope est la boucle V3 ou la boucle GPGR de la glycoprotéine d'enveloppe qp120 d'un lentivirus.

22. Protéine formant des particules selon l'une quelconque des revendications précédentes, dans laquelle une séquence d'acides aminés antigénique est incorporée dans deux des épitopes exposés en surface ou plus.

23. Protéine formant des particules selon la revendication 22, dans laquelle la séquence d'acides aminés antigénique incorporée dans l'un des épitopes exposés en surface est différente de la séquence d'acides aminés antigénique

incorporée dans un autre épitope exposé en surface.

24. Protéine formant des particules selon l'une quelconque des revendications précédentes, dans laquelle plus d'une séquence d'acides aminés antigénique est incorporée dans n'importe quel épitope unique exposé en surface.

25. Protéine formant des particules selon la revendication 24, dans laquelle les séquences d'acides aminés antigéniques incorporées dans n'importe quel épitope unique exposé en surface ne sont pas toutes identiques.

26. Protéine selon la revendication 24 ou la revendication 17, dans laquelle les séquences d'acides aminés antigéniques sont incorporées en tandem au sein de l'épitope exposé en surface.

27. Protéine selon la revendication 23, 25 ou 26, dans laquelle les différentes deuxièmes séquences d'acides aminés sont dérivées de différents épitopes du même antigène.

28. Particule comprenant une pluralité de protéines homologues selon l'une quelconque des revendications 1 à 27.

29. Particule comprenant une pluralité de protéines hétérologues selon l'une quelconque des revendications 1 à 27.

30. Acide nucléique codant pour une protéine de fusion selon l'une quelconque des revendications 1 à 27.

31. Vecteur comportant un acide nucléique selon la revendication 30.

32. Cellule hôte portant un vecteur selon la revendication 31.

33. Cellule hôte selon la revendication 32, où la cellule hôte est E. Coli.

34. Cellule hôte selon la revendication 32, où la cellule hôte est une cellule de levure, par exemple Saccharomyces cerevisiae ou Pichia pastoris.

35. Cellules hôtes selon la revendication 32, où la cellule hôte est une cellule d'insecte, par exemple Spodoptera frugiperda.

36. Anticorps produits ou dirigés contre des antigènes particulaires selon l'une quelconque des revendications 1 à 29.

37. Utilisation de protéines et/ou de particules hybrides selon l'une quelconque des revendications 1 à 29, pour la préparation d'un vaccin immunothérapeutique ou prophylactique.

38. Utilisation d'antigènes particulaires selon les revendications 1 à 29 comme agent de diagnostic.

39. Composition pharmaceutique ou vétérinaire comprenant une protéine selon l'une quelconque des revendications 1 à 29 ainsi qu'un véhicule acceptable sur le plan pharmaceutique et/ou vétérinaire.

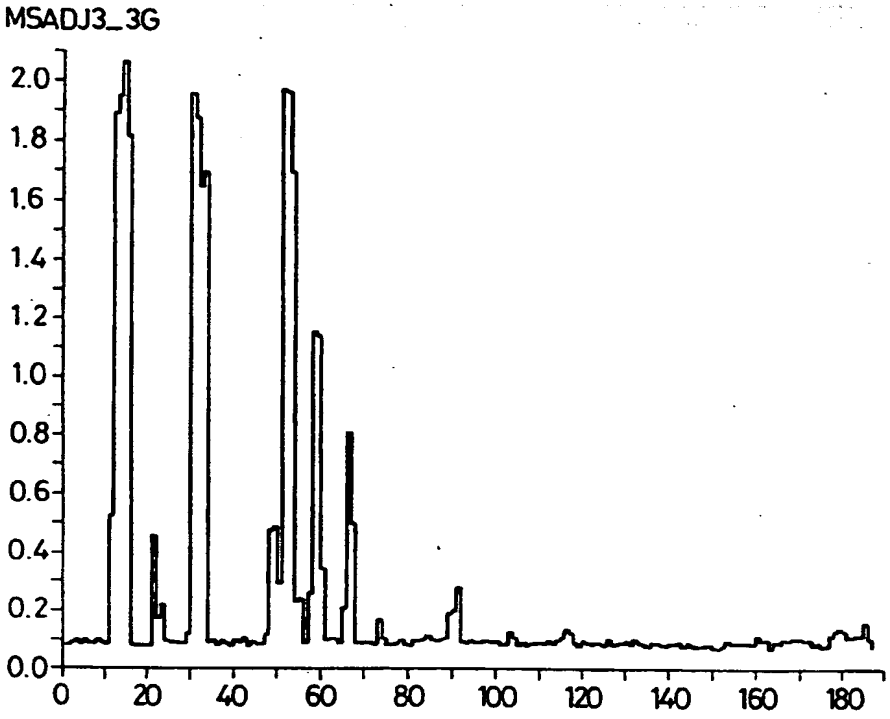
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Fig.1a

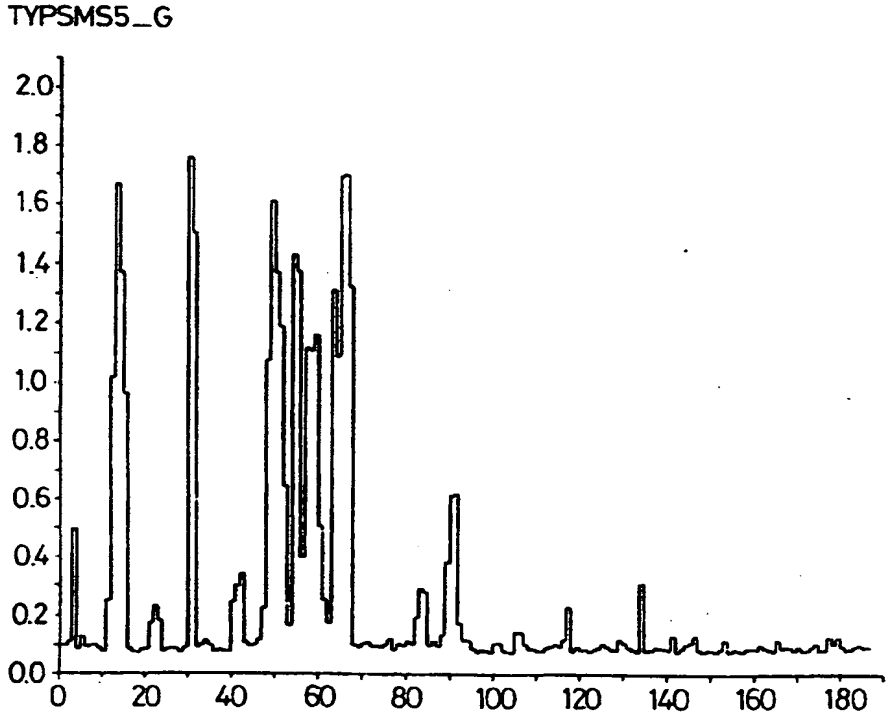
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Fig.1b

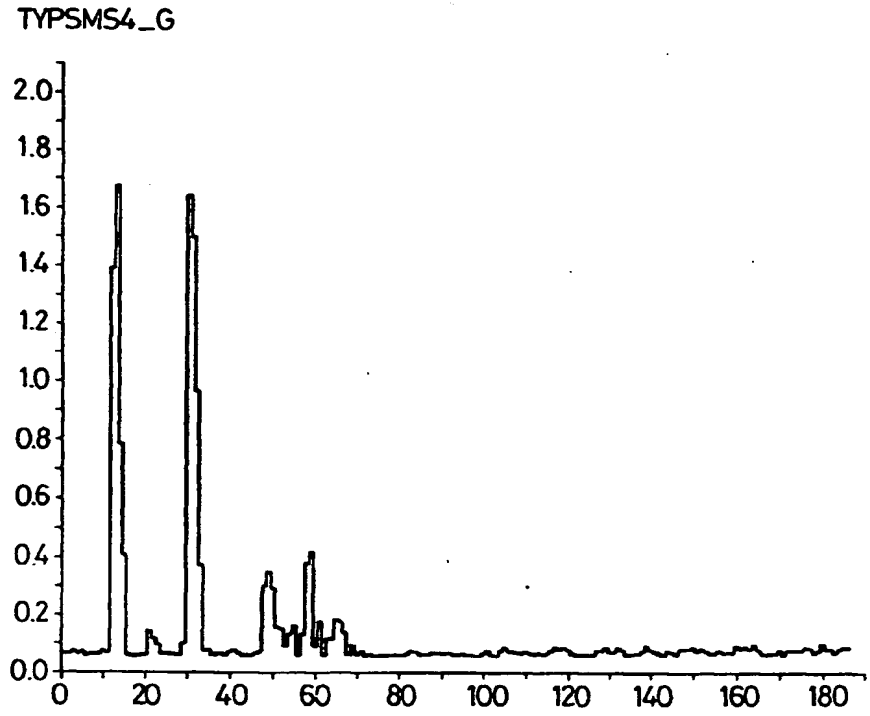
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Fig.1c

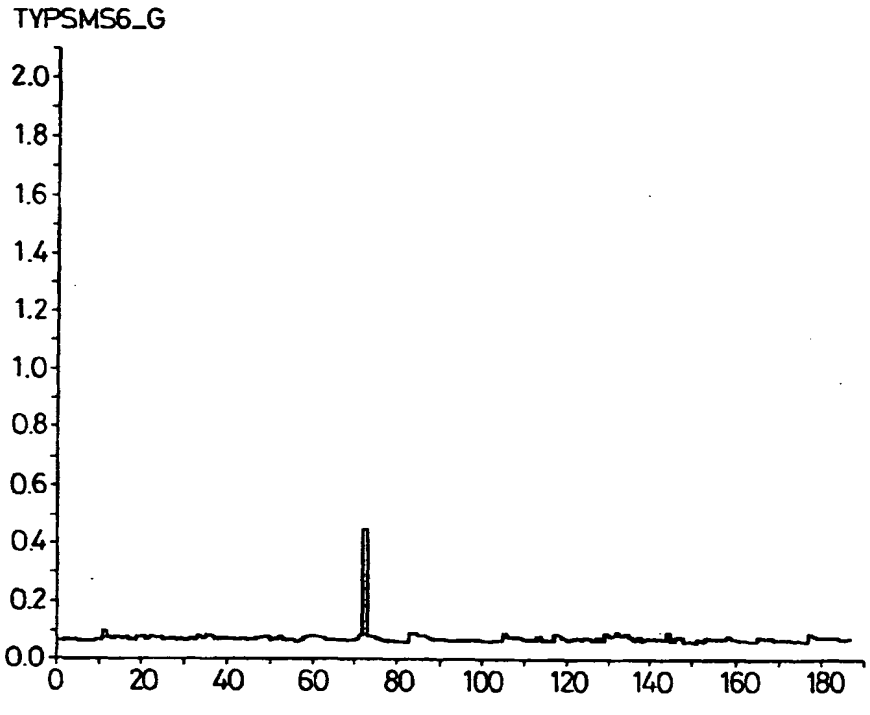
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Fig.1d

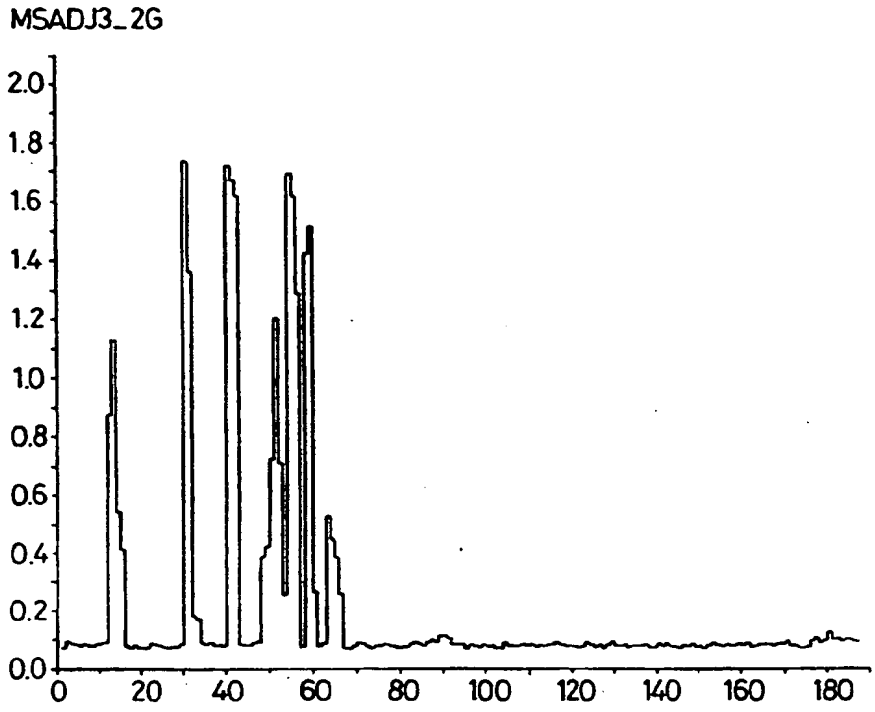
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Fig.1e

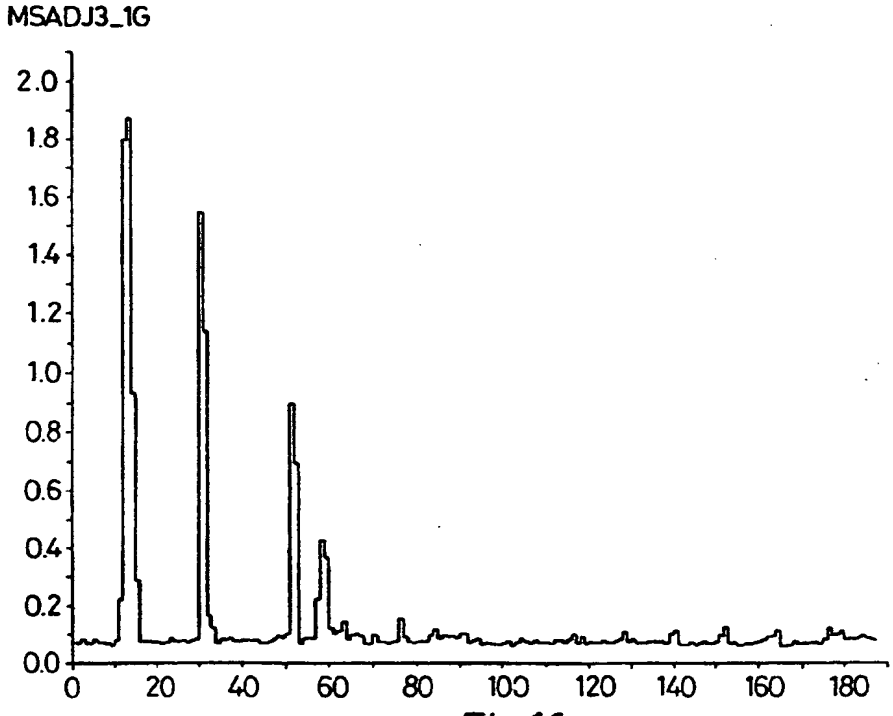
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Fig.1f

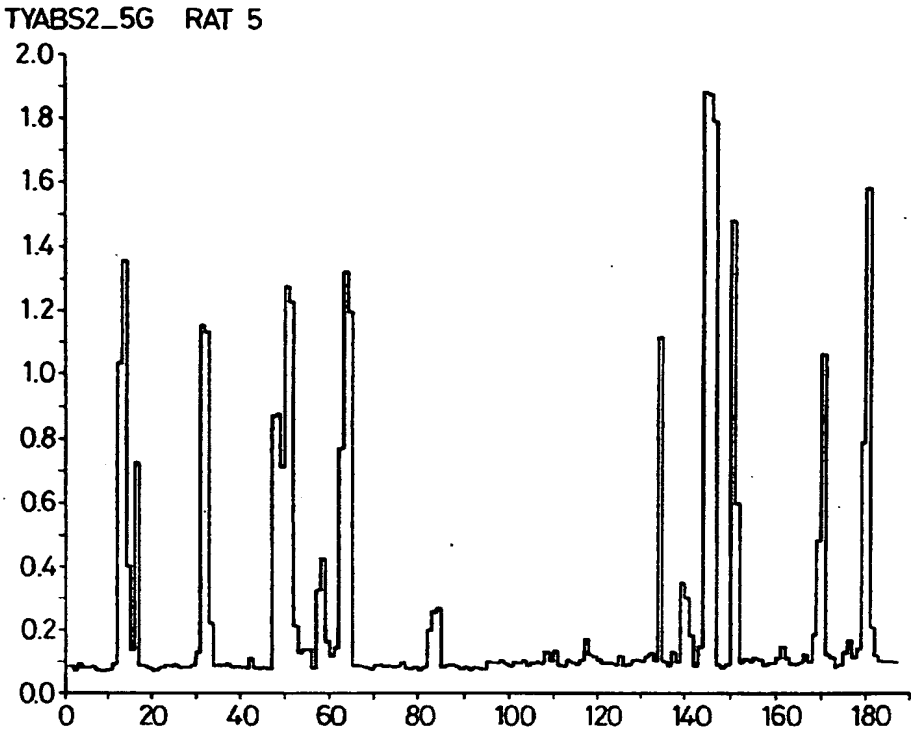

Fig.2a(I)

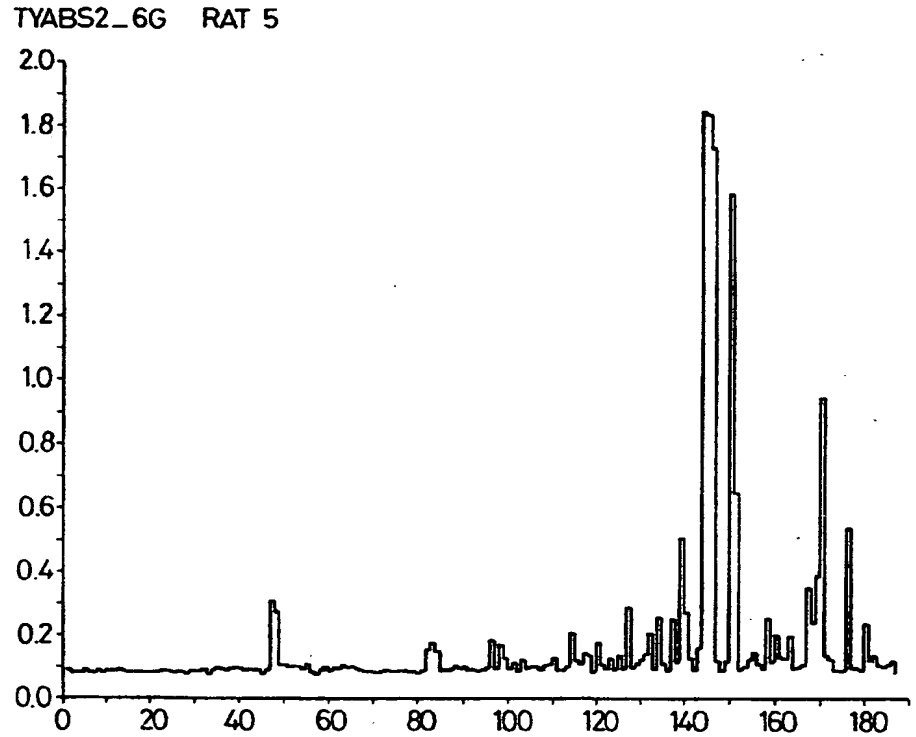

Fig.2a(II)

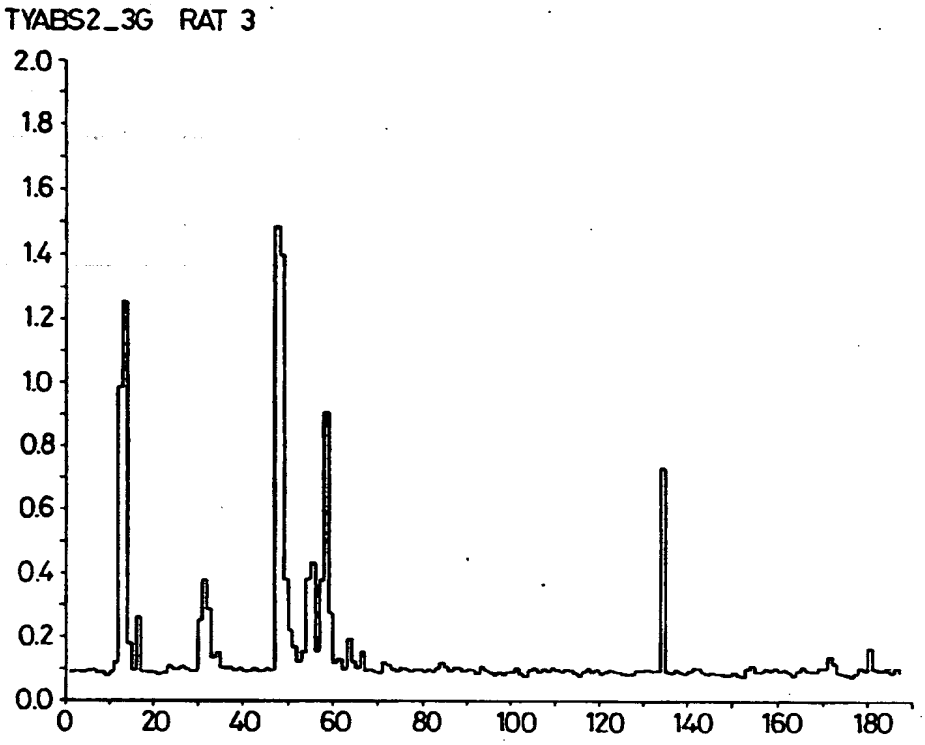

Fig.2b(I)

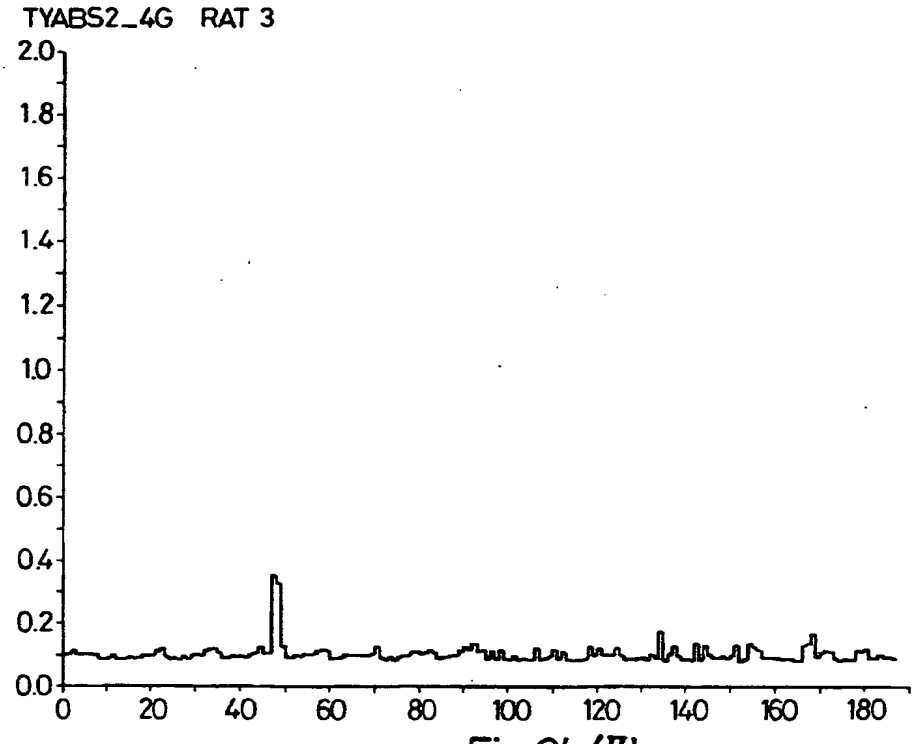

Fig.2b(II)

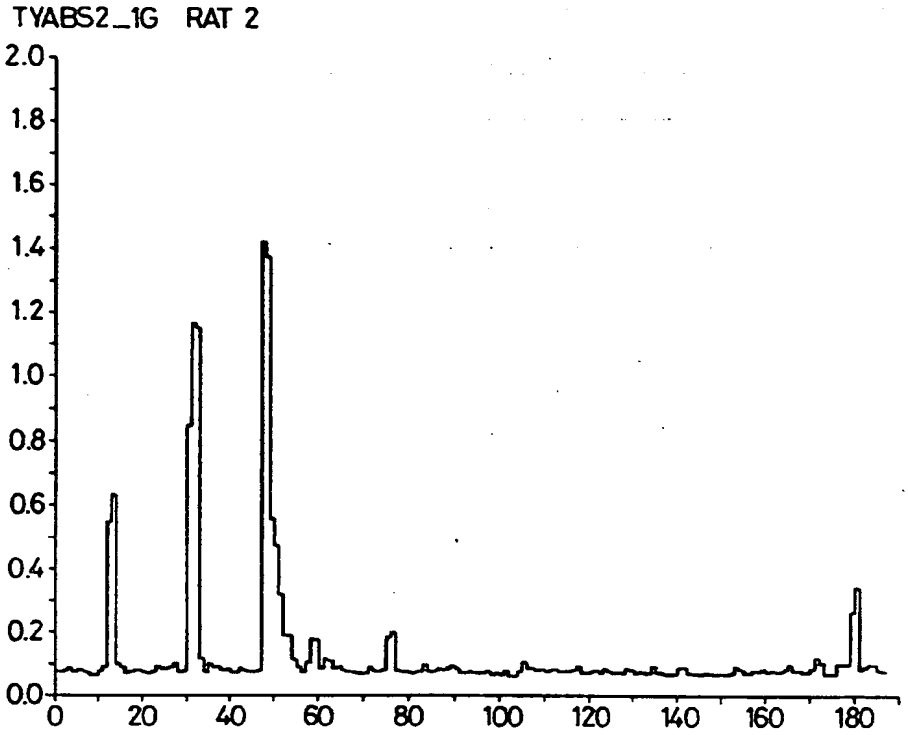

Fig.2c(I)

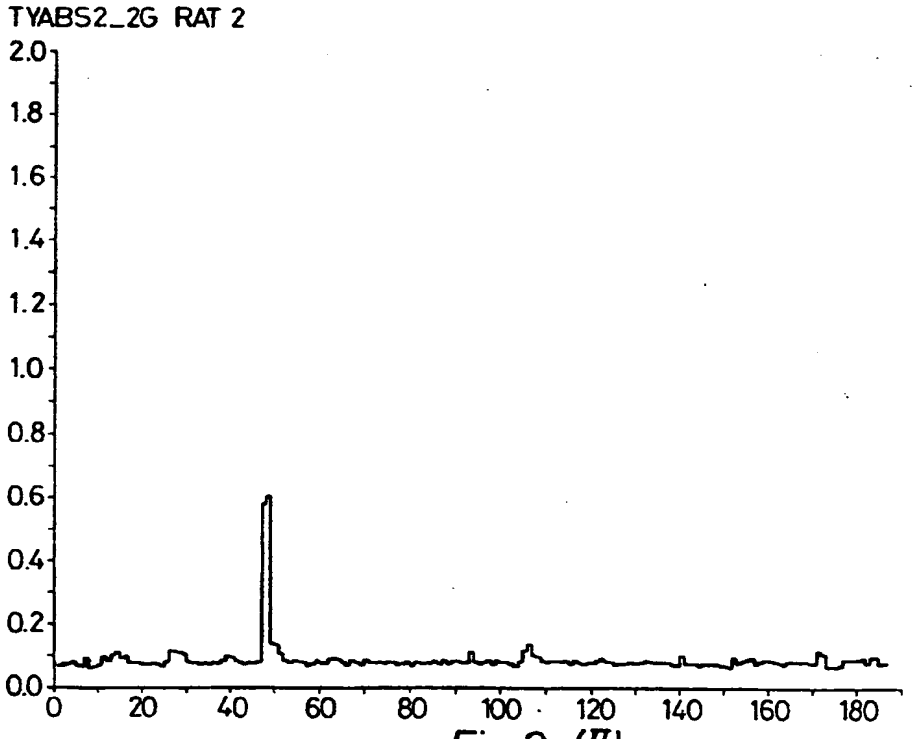

Fig.2c(II)

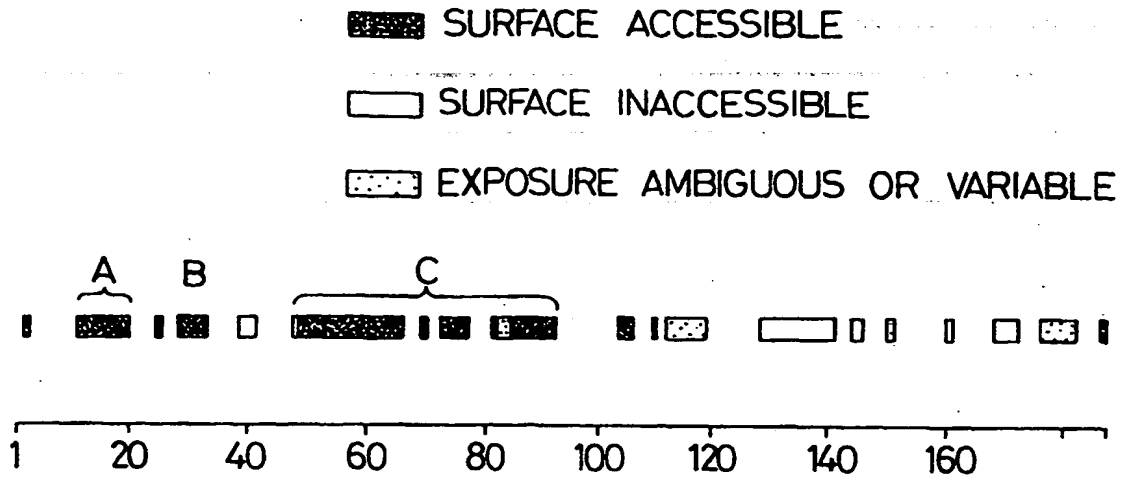
SURFACE ACCESSIBLE
SURFACE INACCESSIBLE
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Fig.3

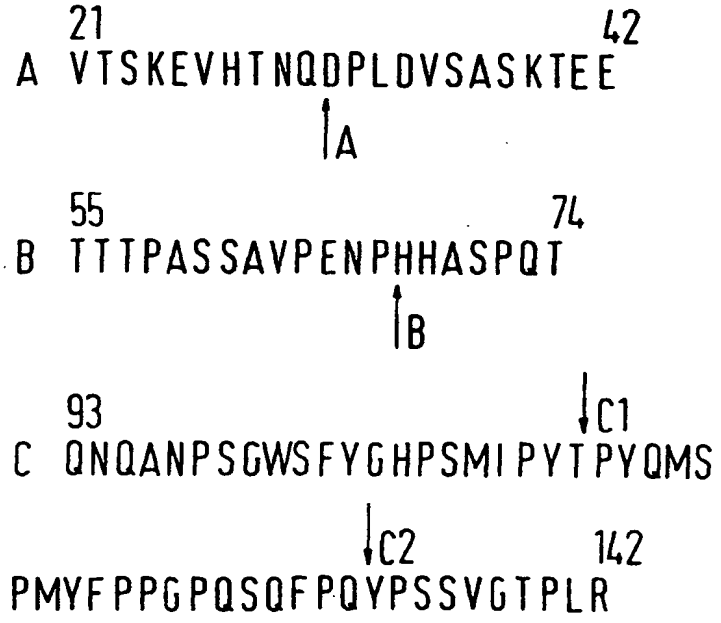
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Fig.4

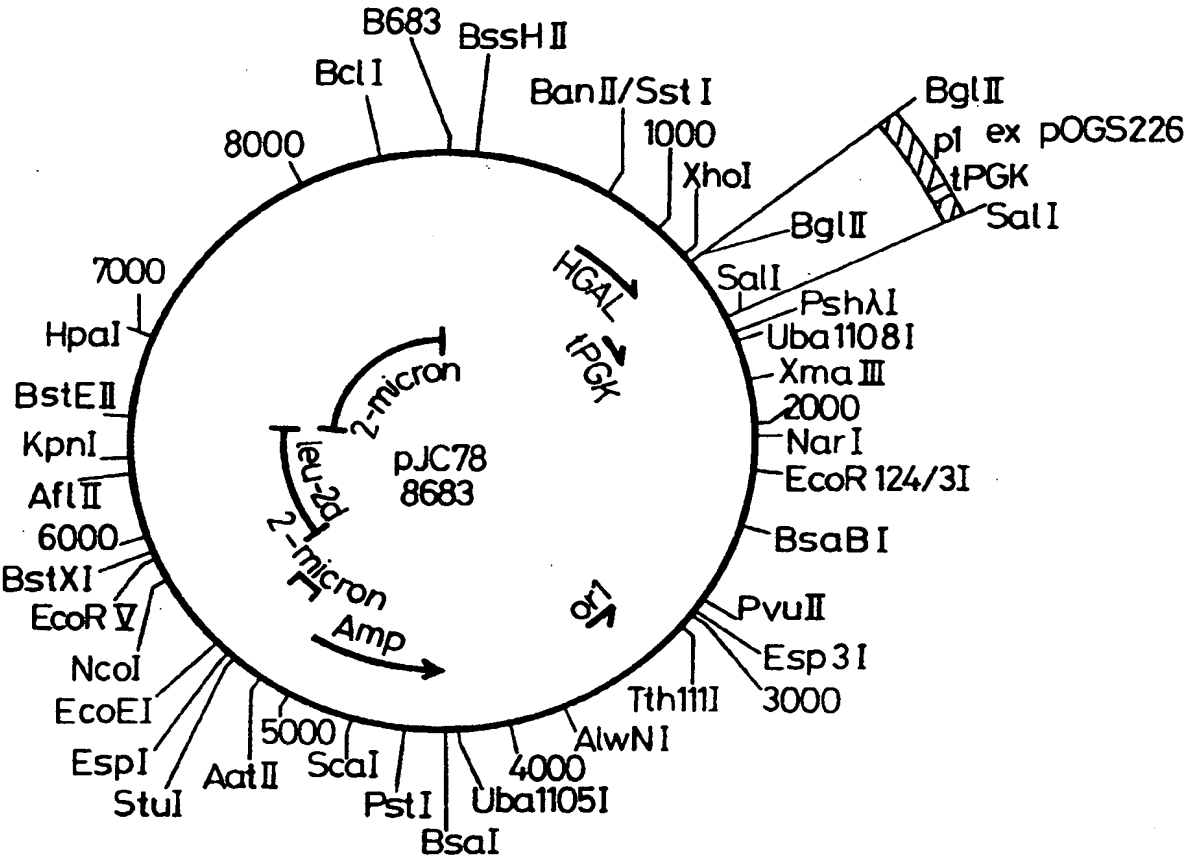
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Fig.5

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(54) Title: MELANOMA ANTIGENS

(57) Abstract

The present invention provides a nucleic acid sequence encoding a melanoma antigen recognized by T lymphocytes, designated MART-1. This invention further relates to bioassays using the nucleic acid sequence, protein or antibodies of this invention to diagnose, assess or prognose a mammal afflicted with melanoma or metastata melanoma. This invention also provides immunogenic peptides derived from the MART-1 melanoma antigen and a second melanoma antigen designated gp100. This invention further provides immunogenic peptides derived from the MART-1 melanoma antigen or gp100 antigen which have been modified to enhance their immunogenicity. The proteins and peptides provided can serve as an immunogen or vaccine to prevent or treat melanoma.

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GA	Gabon				

TITLE OF THE INVENTION

MELANOMA ANTIGENS

This application is a continuation-in-part of United States Patent application 08/231,565 filed on April 22, 1994, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention is in the field of prevention and treatment of human cancers. More specifically, this invention relates to genes encoding melanoma antigens recognized by T-Cells and their corresponding proteins and to preventative, diagnostic and therapeutic applications which employ these genes or proteins.

BACKGROUND OF THE INVENTION

Melanomas are aggressive, frequently metastatic tumors derived from either melanocytes or melanocyte related nevus cells ("Cellular and Molecular Immunology" (1991) (eds) Abbas A.K., Lechtman, A.H., Pober, J.S.; W.B. Saunders Company, Philadelphia: pages 340-341). Melanomas make up approximately three percent of all skin cancers and the worldwide increase in melanoma is unsurpassed by any other neoplasm with the exception of lung cancer in women ("Cellular and Molecular Immunology" (1991) (eds) Abbas, A.K., Lechtiman, A.H., Pober, J.S.; W.B. Saunders Company Philadelphia pages: 340-342; Kirkwood and Agarwala (1993) Principles and Practice of Oncology 7:1-16). Even when melanoma is apparently localized to the skin, up to 30% of the patients will develop systemic metastasis and the majority will die (Kirkwood and Agarwala (1993) Principles and Practice of Oncology 7:1-16). Classic modalities of treating melanoma include surgery, radiation and chemotherapy. In the past decade immunotherapy and gene therapy have emerged as new and promising methods for

treating melanoma.

T cells play an important role in tumor regression in most murine tumor models. Tumor infiltrating lymphocytes (TIL) that recognize unique cancer antigens can be isolated from many murine tumors. The adoptive transfer of these TIL plus interleukin-2 can mediate the regression of established lung and liver metastases (Rosenberg, S.A., et al., (1986) Science 233:1318-1321). In addition, the secretion of IFN-γ by injected TIL significantly correlates with in vivo regression of murine tumors suggesting activation of T-cells by the tumor antigens. (Barth, R.J., et al., (1991) J. Exp. Med. 173:647-658). The known ability of tumor TIL to mediate the regression of metastatic cancer in 35 to 40% of melanoma patients when adoptively transferred into patients with metastatic melanoma attests to the clinical importance of the antigens recognized (Rosenberg, S.A., et al., (1988) N Engl J Med 319:1676-1680; Rosenberg S.A. (1992) J. Clin. Oncol. 10:180-199).

T cell receptors on $CD8^+$ T cells recognize a complex consisting of an antigenic peptide (9-10 amino acids for HLA-A2), ß-2 microglobulin and class I major histocompatibility complex (MHC) heavy chain (HLA-A, B, C, in humans). Peptides generated by digestion of endogenously synthesized proteins are transported into the endoplastic reticulum, bound to class I MHC heavy chain and ß2 microglobulin, and finally expressed in the cell surface in the groove of the class I MHC molecule. Therefore, T cells can detect molecules that originate from proteins inside cells, in contrast to antibodies that detect intact molecules expressed on the cell surface. Therefore, antigens recognized by T cells may be more useful than antigens recognized by antibodies.

Strong evidence that an immune response to cancer exists in humans is provided by the existence of lymphocytes within melanoma deposits. These lymphocytes,

when isolated, are capable of recognizing specific tumor antigens on autologous and allogeneic melanomas in an MHC restricted fashion. (Itoh, K. et al. (1986), Cancer Res. 46: 3011-3017; Muul, L.M., et al. (1987), J. Immunol. 138:989-995); Topalian, S.L., et al., (1989) J. Immunol. 142: 3714-3725; Darrow, T.L., et al., (1989) J. Immunol. 142: 3329-3335; Hom, S.S., et al., (1991) J. Immunother. 10:153-164; Kawakami, Y., et al., (1992) J. Immunol. 148: 638-643; Hom, S.S., et al., (1993) J. Immunother. 13:18-30; O'Neil, B.H., et al., (1993) J. Immunol. 151: 1410-1418). TIL from patients with metastatic melanoma recognize shared antigens including melanocyte-melanoma lineage specific tissue antigens in vitro (Kawakami, Y., et al., (1993) J. Immunother. 14: 88-93; Anichini, A. et al., (1993) et al., J. Exp. Med. 177: 989-998). Anti-melanoma T cells appear to be enriched in TIL probably as a consequence of clonal expansion and accumulation at the tumor site in vivo (Sensi, M., et al., (1993) J. Exp. Med. 178:1231-1246). The fact that many melanoma patients mount cellular and humoral responses against these tumors and that melanomas express both MHC antigens and tumor associated antigens (TAA) suggests that identification and characterization of additional melanoma antigens will be important for immunotherapy of patients with melanoma.

Peripheral blood lymphocytes have been used to identify potential melanoma tumor antigens. Van Der Bruggen et al. (1991) Science 254: 1643-1647 has characterized a gene coding for a melanoma antigen, designated MAGE-1, using T cell clones established from the peripheral blood of patients who were repetitively immunized in vivo with mutagenized tumor cells. Cytotoxic T-cells derived from the peripheral blood lymphocytes of patients with melanoma were used to identify a potential antigenic peptide encoding MAGE-1 (Traversari, C., et al. (1992) J. Exp. Med. 176:1453-1457). Brichard et al. (1993) J. Exp. Med. 178:489-495 has also characterized a

gene encoding a melanoma antigen designated tyrosinase using peripheral blood lymphocytes from patients who were sensitized by repetitive in vitro stimulation with tumor. Further support for the therapeutic potential of melanoma antigens is provided by Brown et al. (United States Patent No. 5,262,177). Brown et al. (United States Patent Number 5,262,177) relates to a recombinant vaccinia virus-based melanoma vaccine where the melanoma antigen p97 is reported to show a protective effect from tumor cell challenge in a murine model. Characterization of additional melanoma antigens is important for the development of new strategies for cancer immunotherapy, in particular for melanoma.

SUMMARY OF THE INVENTION

This invention relates, in general, to nucleic acid sequences encoding melanoma antigens recognized by T-lymphocytes (MART-1) and protein and peptides encoded by these sequences. This invention further provides bioassays for these nucleic acid sequences, proteins and peptides. This invention also provides peptides which have been derived from the MART-1 amino acid sequence and modified to enhance their immunogenocity. This invention also provides therapeutic uses for the nucleic acid sequences, proteins, peptides or modified peptides described herein.

It is a general object of the present invention to provide a substantially purified and isolated nucleic acid sequence which encodes for the MART-1 melanoma antigen.

It is another object of this invention to provide a recombinant molecule comprising a vector and all or part of the nucleic acid sequence encoding MART-1.

It is another object of this invention to produce recombinant proteins encoded by all or part of the nucleic acid sequence encoding MART-1.

It is a further object of this invention to provide monoclonal or polyclonal antibodies reactive with the

MART-1 protein, peptides or portions thereof.

It is an object of this invention to provide methods of detecting the MART-1 gene or MART-1 mRNA in a biological sample.

It is another object of this invention to provide methods of detecting the MART-1 protein or peptides in a biological sample.

It is an object of this invention to provide diagnostic methods for human disease, in particular for melanomas and metastatic melanomas.

It is a further object of this invention to provide methods for prophylactic or therapeutic uses involving all or part of the nucleic acid sequence encoding MART-1 and its corresponding protein or peptides derived from the MART-1 amino acid sequence.

It is also an object of this invention to provide melanoma vaccines comprising all or part of the nucleic acid sequence encoding MART-1 or its corresponding protein for preventing or treating melanoma.

It is a further object of this invention to provide immunogenic peptides derived from the MART-1 protein sequence for use in vaccines.

It is yet another object of this invention to provide peptides derived from the MART-1 protein sequence which have been modified to increase their immunogenicity or enhance induction of antimelanoma immune response by enhancing their binding to MHC molecules, for use in the prophylactic or therapeutic methods described herein.

In addition, it is another object of this invention to provide multivalent vaccines comprising all or part of the MART-1 nucleic acid sequence or its corresponding protein or peptides and at least one other immunogenic molecule capable of eliciting the production of antibodies in a mammal to melanoma antigens.

It is another object of this invention to provide a method for preventing or treating melanoma utilizing all

or part of the MART-1 nucleic acid sequence or its corresponding protein in gene therapy protocols.

It is a further object of this invention to provide immunogenic peptides derived from gp100 melanoma antigen protein sequences for use in vaccines.

It is yet another object of this invention to provide peptides derived from gp100 melanoma antigen sequences which have been modified to increase their immunogenicity or enhance induction of antimelanoma immune response by enhancing binding to MHC molecules for use in the prophylactic and therapeutic methods described herein .

It is yet another object of this invention to provide a method of prophylactic or therapeutic immunization for melanoma using the vaccines described herein.

It is a further object of this invention to provide a method of identifying melanoma antigens that would constitute potential targets for immunotherapy.

It is yet another object of this invention to provide a method of identifying candidate immunogenic peptides derived from either the MART-1 or gp100 sequences for use in immunotherapy.

DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide and predicted amino acid sequence of the cDNA encoding the MART-1 antigen. The hydrophobic region is underlined.

Figures 2 and 2B show titration of MART-1 peptides for recognition by TIL. T2 cells were incubated with varied concentrations of the purified MART-1 peptides, M9-1, M9-2, M9-3, M10-2, M10-3, M10-4 and M10-5, and lysis by TIL clone A42 (Figure 2A) or TIL line TIL1235 (Figure 2B)was measured by 4h-^{51}Cr release cytotoxicity assay at an E (EFFECTOR):T (TARGET) ratio of 20:1 for A42 and 40:1 for TIL1235. Peptide M9-2 sensitized T2 cells at a concentration of 1ng/ml. The purified peptide M10-4 was recognized by TIL1235, but not by A42. (M9-1 |-|,

M9-2 ●-●, M9-3 ■-■ , M10-2 ▲-▲ , M10-

3 ▼-▼ , M10-4 ■-■ , M10-5 +-+).

Figure 3A shows a radionuclide scan of patient 1200 with metastatic melanoma after receiving the adoptive transfer of autologous ^{111}In labeled TIL1200. The arrow indicates one of the areas of TIL accumulation corresponding to a metastatic lesion in the left thigh.

Figure 3B shows regression of subcutaneous metastatic tumors following treatment with TIL1200 plus IL-2. Treatment began on day 0.

Figures 4A and 4B show the nucleic acid sequence of the full length cDNA25. The start and stop codons are underlined.

Figure 5A shows amino acid sequence of the full length cDNA25. The antigenic peptide is underlined.

Figure 5B shows comparison of the amino acid sequence of the full length cDNA25 (cDNA25FL), the truncated form of cDNA25 (cDNA25TR), Pmel17, ME20 and gp100. (•indicates deletion; - indicates identity).

Figures 6A, 6B and 6C show northern blot analysis of melanoma (Figure 6A) and neonatal melanocyte cell lines (Figure 6B) and various fresh tissues (Figure 6C) (10-20ug of total RNA) with a cDNA25 probe(the Sal I digested fragment of pCRII-cDNA25) and the ß-actin probe(Clontech). C32, 586mel melanoma cell lines and NHEM529, NHEM530 neonatal melanocyte cell lines were very weak positive.

Figures 7A-7B show the location of gp100 epitopes and the DNA fragments tested for epitope analysis and recognition by CTL. Figure 7A. Five DNA fragments (D3, D5, D4, C4, 25TR) tested for epitope analysis are shown (---,identical amino acid). Locations of the identified epitopes are underlined. Figure 7B. Recognition by CTL (620-1, 620-2, 660-1, 1143, 1200) of COS 7 cells transfected with each DNA fragment in pcDNA3 plasmid along with HLA-A2.1 cDNA by IFN-γ secretion assays are shown (+, recognized; -, not recognized).

Figures 8A-8D show titration of gp100 epitopes by

sensitization of HLA-A2.1+ T2 cells for CTL lysis. Lysis of T2 cells preincubated with peptides was tested in a 4h ^{51}Cr release cytotoxicity assay. Figure 8A, Lysis by TIL1200 of T2 cells incubated with $G9_{154}$(■) or $G10_{154}$(●). Figure 8B. Lysis by TIL620 of T2 cells incubated with $G9_{209}$(■) or $G10_{208}$(●). Figure 8C. Lysis by TIL660-1 of T2 cells incubated with $G9_{280}$(■). Figure 8D. Lysis by TIL660-2 of T2 cells incubated with G10-5(■).

DETAILED DESCRIPTION OF THE INVENTION

For the purpose of a more complete understanding of the invention, the following definitions are described herein. Nucleic acid sequences includes, but is not limited to, DNA, RNA or cDNA. Nucleic acid sequence as used herein refers to an isolated and purified nucleic acid sequence. MART-1 messenger RNA (mRNA) refers to one or more RNA transcripts which are a product of the MART-1 gene. Substantially homologous as used herein refers to substantial correspondence between the nucleic acid sequence of MART-1 shown in Figure 1 (SEQ ID NO:1) and that of any other nucleic acid sequence. Substantially homologous means about 50-100% homologous homology, preferably by about 70-100% homology, and most preferably about 90-100% homology between the MART-1 sequence and that of any other nucleic acid sequence. In addition, substantially homologous as used herein also refers to substantial correspondences between the amino acid sequence of the MART-1 antigen shown in Figure 1 (SEQ ID NO:2) and that of any other amino acid sequence.

Major Histocompatibility Complex (MHC) is a generic designation meant to encompass the histo-compatibility antigen systems described in different species including the human leucocyte antigens (HLA).

The term melanoma includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes or melanocytes related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas,

melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by, chromosomal abnormalities, degenerative growth and developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections, inappropriate tissue expression of a gene, alterations in expression of a gene, or carcinogenic agents. The aforementioned melanomas can be diagnosed, assessed or treated by methods described in the present application.

By atypical mole we mean a mole with features that are abnormal and may be precancerous.

By melanoma antigen or immunogen we mean all or parts thereof of the MART-1 protein or peptides based on the MART-1 protein sequence capable of causing a cellular or humoral immune response in a mammal. Such antigens may also be reactive with antibodies from animals immunized with all, part or parts of the MART-1 protein (SEQ ID NO:2). Such a protein or peptide may be encoded by all or part of the MART-1 nucleic acid sequence of this invention.

By immunogenic peptide we mean a peptide derived from the MART-1 protein sequence or a gp100 protein sequence capable of causing a cellular or humoral immune response in a mammal. Such peptides may be reactive with antibodies from an animal immunized with the peptides. Such peptides are about 5-20 amino acid in length preferably about 8 to 15 amino acids in length, and most preferably about 9-10 amino acids in length.

One skilled in the art will understand that the bioassays of the present invention may be used in the analysis of biological samples or tissues from any vertebrate species. In a preferred embodiment, mammalian biological samples or tissues are analyzed.

Tissue includes, but is not limited to, single cells,

whole organs and portions thereof. Biological samples include, but are not limited to, tissues, primary cultures of mammalian tissues, biopsy specimens, pathology specimens, and necropsy specimens. Mammal includes but is not limited to, humans, monkeys, dogs, cats, mice, rats, pigs, cows, pigs, horses, sheep and goats.

The present invention provides a nucleic acid sequence which encodes a novel melanoma antigen recognized by T cells. This novel melanoma antigen designated MART-1 (melanoma antigen recognized by T-Cells-1). MART-1 shows no significant homology to any known melanoma antigen and thus represents a new melanoma antigen. The MART-1 antigen contains a highly hydrophobic region from amino acids 27 to 47 (SEQ ID. NO:2) followed by three arginine residues, suggestive of a transmembrane protein. Although no significant homology exists to the entire protein there is a 27 amino acid segment (amino acids 57-83; SEQ ID. NO:2) that is 37% identical to a Type II membrane protein previously recognized as mouse natural killer cell surface protein NKR-P1 (Yokoyama, W.M., et al. (1991), J. Immunol. 147:3229-3236). MART-1 does not contain a leader sequence characteristic of many Type I membrane proteins (Singer, S.J. (1990) Annu. Rev. Cell Biol. 6: 247-296).

MART-1 RNA expression appears to be restricted to fresh and cultured melanoma and melanocyte cell lines and human retina; expression has not been found in any other fresh or cultured tissues or other tumor histologies tested. The cDNA sequence for MART-1 is shown in Figure 1 (SEQ ID NO:1), the deduced amino acid sequence for the MART-1 protein is also shown in Figure 1 (SEQ ID NO.:1).

The nucleic acid sequence for MART-1 shown in Figure 1 (SEQ ID NO.:1), represents a preferred embodiment of the invention. It is, however, understood by one skilled in the art that due to the degeneracy of the genetic code variations in the cDNA sequence shown in Figure 1 (SEQ ID NO.:1) will still result in a DNA sequence capable of

encoding the MART-1 protein antigen. Such DNA sequences are therefore functionally equivalent to the sequence set forth in Figure 1 (SEQ ID NO.:1) and are intended to be encompassed within the present invention. Further, a person of skill in the art will understand that there are naturally occurring allelic variations in a given species of the MART-1 nucleic acid sequence shown in Figure 1 (SEQ ID NO.:1), these variations are also intended to be encompassed by the present invention.

The predicted MART-1 antigen is a 118 amino acid protein of about 13 (kd). This invention further includes MART-1 protein or peptides or analogs thereof having substantially the same function as the MART-1 antigen or protein of this invention. Such proteins or polypeptides include, but are not limited to, a fragment of the protein, or a substitution, addition or deletion mutant of the MART-1 protein. This invention also encompasses proteins or peptides that are substantially homologous to the MART-1 antigen. Substantially homologous means about 50-100% homology, preferably about 70-100% homology, and most preferably about 90-100% homology between the MART-1 and any another amino acid sequence or protein or peptide.

The term "analog" includes any polypeptide having an amino acid residue sequence substantially identical to the MART-1 sequence specifically shown herein (Figure 1; SEQ ID NO: 1) in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the functional aspects of the MART-1 antigen as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine

or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid or another.

The phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue. "Chemical derivative" refers to a subject polypeptide having one or more residues chemically derivatized by reaction of a functional side group. Examples of such derivatized molecules include for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those proteins or peptides which contain one or more naturally-occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Proteins or polypeptides of the present invention also include any polypeptide having one or more additions and/or deletions or residues relative to the sequence of a polypeptide whose sequence is encoded is the DNA of MART-1, so long as the requisite activity is maintained.

This invention also provides a recombinant DNA molecule comprising all or part of the MART-1 nucleic acid sequence (SEQ ID NO: 1) and a vector. Expression vectors suitable for use in the present invention may comprise at least one expression control element operationally linked

to the nucleic acid sequence. The expression control elements are inserted in the vector to control and regulate the expression of the nucleic acid sequence. Examples of expression control elements include, but are not limited to, lac system, operator and promoter regions of phage lambda, yeast promoters and promoters derived from polyoma, adenovirus, retrovirus or SV40. Additional preferred or required operational elements include, but are not limited to, leader sequence, termination codons, polyadenylation signals and any other sequences necessary or preferred for the appropriate transcription and subsequent translation of the nucleic acid sequence in the host system. It will be understood by one skilled in the art the correct combination of required or preferred expression control elements will depend on the host system chosen. It will further be understood that the expression vector should contain additional elements necessary for the transfer and subsequent replication of the expression vector containing the nucleic acid sequence in the host system. Examples of such elements include, but are not limited to, origins of replication and selectable markers. It will further be understood by one skilled in the art that such vectors are easily constructed using conventional methods (Ausubel et al., (1987) in "Current Protocols in Molecular Biology", John Wiley and Sons, New York, New York) or commercially available.

Another aspect of this invention relates to a host organism into which recombinant expression vector containing all or part of the MART-1 nucleic acid sequence has been inserted. The host cells transformed with the MART-1 nucleic acid sequence of this invention include eukaryotes, such as animal, plant, insect and yeast cells and prokaryotes, such as E. coli. The means by which the vector carrying the gene may be introduced into the cell include, but are not limited to, microinjection, electroporation, transduction, or transfection using DEAE-

dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (1989) in "Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York).

In a preferred embodiment, eukaryotic expression vectors that function in eukaryotic cells are used. Examples of such vectors include, but are not limited to, retroviral vectors, vaccinia virus vectors, adenovirus vectors, herpes virus vector, fowl pox virus vector, bacterial expression vectors, plasmids, such as pcDNA3 (Invitrogen, San Diego, CA) or the baculovirus transfer vectors. Preferred eukaryotic cell lines include, but are not limited to, COS cells, CHO cells, HeLa cells, NIH/3T3 cells, 293 cells (ATCC# CRL1573), T2 cells, dendritic cells, or monocytes. In a particularly preferred embodiment the recombinant MART-1 protein expression vector is introduced into mammalian cells, such as NIH/3T3, COS, CHO, 293 cells (ATCC #CRL 1573), T2 cells, dendritic cells, or monocytes to ensure proper processing and modification of the MART-1 protein. In an alternative embodiment the MART-1 cDNA is introduced into COS7 (Gluzman, Y. et al. (1981) Cell 23: 175-182). The choice of an appropriate cell is within the skill of a person in the art.

In one embodiment the expressed recombinant MART-1 protein may be detected by methods known in the art which include Coomassie blue staining and Western blotting using antibodies specific for the MART-1 protein.

In a further embodiment, the recombinant protein expressed by the host cells can be obtained as a crude lysate or can be purified by standard protein purification procedures known in the art which may include differential precipitation, molecular sieve chromatography, ion-exchange chromatography, isoelectric focusing, gel electrophoresis, affinity, and immunoaffinity chromatography and the like. (Ausubel et. al., (1987) in

"Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York). In the case of immunoaffinity chromatography, the recombinant protein may be purified by passage through a column containing a resin which has bound thereto antibodies specific for the MART-1 protein (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York).

The nucleic acid sequence or portions thereof, of this invention are useful as probes for the detection of expression of the MART-1 gene in normal and diseased tissue. Therefore, another aspect of the present invention relates to a bioassay for detecting messenger RNA encoding the MART-1 protein in a biological sample comprising the steps of (a) contacting all or part of the nucleic acid sequence of this invention with said biological sample under conditions allowing a complex to form between said nucleic acid sequence and said messenger RNA, (b) detecting said complexes and, (c) determining the level of said messenger RNA.

RNA can be isolated as whole cell RNA or as poly(A)$^+$ RNA. Whole cell RNA can be isolated by a variety of methods known to those skilled in the art. (Ausubel et al., (1987) on "Current Protocols in Molecular Biology", John Wiley and Sons, New York). Such methods include extraction of RNA by differential precipitation (Birnboim, H.C. (1988) Nucleic Acids Res., 16:1487-1497), extraction of RNA by organic solvents (Chomczynski, P. et al. (1987) Anal. Biochem., 162:156-159) and the extraction of RNA with strong denaturants (Chirgwin, J.M. et al. (1979) Biochemistry, 18:5294-5299). Poly(A)$^+$ RNA can be selected from whole cell RNA by affinity chromatography on oligo-d(T) columns (Aviv, H. et al. (1972) Proc. Natl. Acad. Sci., 69:1408-1412). Examples of methods for determining cellular messenger mRNA levels for step (c) include, but are not limited to Northern blotting (Alwine, J.C. et al.

(1977) Proc. Natl. Acad. Sci., 74:5350-5354), dot and slot hybridization (Kafatos, F.C. et al. (1979) Nucleic Acids Res., 7:1541-1522), filter hybridization (Hollander, M.C. et al. (1990) Biotechniques; 9:174-179), RNase protection (Sambrook et. al., (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, NY), polymerase chain reaction (Watson, J.D. et al. (1992) in "Recombinant DNA" Second Edition, W.H. Freeman and Company, New York) and nuclear run-off assays (Ausubel et. al., (1987) in "Current Protocols in Molecular Biology" Supplement 9 (1990); John Wiley and Sons, New York, New York).

Detection of complexes in Step (b) of the bioassay can also be carried out by a variety of techniques. Detection of the complexes by signal amplification can be achieved by several conventional labelling techniques including radiolabels and enzymes (Sambrook et. al., (1989) in "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, New York; Ausubel et al., (1987) in "Current Protocols in Molecular Biology, John Wiley and Sons, New York New York). Radiolabelling kits are also commercially available. The MART-1 nucleic acid sequence used as a probe in step(c) of the bioassay may be RNA or DNA. Preferred methods of labelling the DNA sequences are with ^{32}P using Klenow enzyme or polynucleotide kinase. Preferred methods of labeling RNA or riboprobe sequences are with ^{32}P or ^{35}S using RNA polymerases. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R.N.K. et al. (1973) Proc. Natl. Acad. Sci., 70:2238-2242; Heck, R.F. (1968) S. Am. Chem. Soc., 90:5518-5523), methods which allow detection by chemiluminescence (Barton, S.K. et al. (1992) J. Am. Chem. Soc., 114:8736-8740) and methods utilizing biotinylated nucleic acid probes (Johnson, T.K. et al. (1983) Anal.

Biochem., 133:125-131; Erickson, P.F. et al. (1982) J. of Immunology Methods, 51:241-249; Matthaei, F.S. et al (1986) Anal. Biochem., 157:123-128) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labelling kits are also commercially available.

Examples of biological samples that can be used in this bioassay include, but are not limited to, primary mammalian cultures, continuous mammalian cell lines, such as melanocyte cell lines, mammalian organs such as skin or retina, tissues, biopsy specimens, neoplasms, pathology specimens, and necropsy specimens.

In a preferred embodiment, a ^{32}P radiolabelled MART-1 probe, as exemplified in Example 1, is used. Preferably the MART-1 probe is the full length cDNA in Figure 1 (SEQ ID NO:1). The approximately 1.6 Kilobase (kb) cDNA (Figure 1; SEQ ID NO:1) was cloned into the vector and the resulting plasmid deposited with the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA on April 14, 1994, and given ATCC Deposit Number 75738. The full length MART-1 nucleic acid sequence can be isolated from the pCRII plasmid by digestion with HINDIII and XhoI restriction enzymes. This 1.6kb nucleic acid sequence can then be used as a probe. This probe is used to detect MART-1 mRNA in total RNA or poly A^+ RNA isolated from a variety of tissues or biological samples.

In another embodiment, combinations of oligonucleotide pairs based on the MART-1 sequence in Figure 1 (SEQ ID NO.:1) are used as Polymerase Chain Reaction (PCR) primers to detect MART-1 mRNA in a biological sample. These primers can be used in a method following the reverse transcriptase - Polymerase Chain Reaction (RT-PCR) process for amplifying selected RNA nucleic acid sequences as detailed in Ausubel et al., (eds) (1987) In "Current Protocols in Molecular Biology" Chapter 15, John Wiley and Sons, New York, New York. The

oligonucleotides can be synthesized by automated instruments sold by a variety of manufacturers or can be commercially prepared based upon the nucleic acid sequence of this invention. One skilled in the art will know how to select PCR primers based on the MART-1 nucleic acid sequence for amplifying MART-1 RNA in a sample.

The MART-1 nucleic acid sequence or portions thereof (Figure 1: SEQ ID NO:1) of this invention are useful to detect alterations of the MART-1 gene in normal or diseased mammalian tissue. By alteration, we mean additions, deletions, substitutions or duplications in the MART-1 gene sequence or gene amplification of the MART-1 gene sequence. Therefore, another aspect of the present invention relates to an assay for detecting alterations of the MART-1 gene in a biological sample comprising the steps of (a) contacting all or part of the nucleic acid sequence of this invention with genomic DNA isolated from a biological sample under conditions allowing a complex to form between said nucleic acid sequence and said genomic DNA, (b) detecting said complexes, and (c) determining alterations in said MART-1 gene by comparison to a control sample.

Standard methods for isolating DNA from a biological sample, detecting alterations in a gene and detecting complex between the MART-1 nucleic acid probe and genomic DNA sequences are provided in manuals such as Sambrook et al., (eds) (1989) "Molecular Cloning, A Laboratory Mineral", Cold Spring Harbor Press, Plainview, New York and in Ausubel et al., (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York.

The MART-1 nucleic acid sequences of this invention (Figure 1; SEQ ID No:1) can also be used as probes to isolate the MART-1 homologs in other species. In a preferred embodiment the MART-1 cDNA (Figure 1; SEQ ID No:1) is used to screen a mammalian cDNA library, positive

clones are selected and sequenced. Examples of tissue sources from which the cDNA library can be synthesized include, but are not limited to skin, retina, melanocytes, neonatal skin and embryos. Preferably a melanoma library is screened using the MART-1 cDNA as a probe (Figure 1; SEQ ID No. 1). One skilled in the art will understand the appropriate hybridization conditions to be used to detect the homologs. Conventional methods for nucleic acid hybridization, construction of libraries and cloning techniques are described in Sambrook et al., (eds) (1989) In "Molecular Cloning A Laboratory Manual" Cold Spring Harbor Press, Plainview, New York and Ausubel et al., (eds) in "Current Protocols in Molecular Biology" (1987), John Wiley and Sons, New York, New York.

It is known that all or parts thereof of the MART-1 protein is an antigen present on melanoma cells. It is therefore another aspect of this invention to provide MART-1 nucleic acid probes to be utilized in detecting MART-1 RNA or alterations in the level of MART-1 mRNA in biological sample isolated from a mammal afflicted with a disease. Examples of such diseases, include but are not limited to, melanomas. By alterations in the level of MART-1 mRNA we mean an increase or decrease in the level of an RNA relative to a control sample or the appearance or disappearance of the MART-1 mRNA relative to a control sample. Detection in the alterations of MART-1 mRNA will allow for diagnosis or the assessment of the diseased state. Therefore, alterations in the level of MART-1 mRNA may be predictive of the prognosis for the afflicted mammal.

In another embodiment the nucleic acid of this invention can be used in <u>in situ</u> hybridization on mammalian tissues to determine the precise site or subcellular site of expression of the MART-1 gene within a tissue. A preferred method of labeling the MART-1 nucleic acid sequence is synthesizing a ^{35}S - labeled RNA probe by

in vitro transcription utilizing SP6 polymerase. In the MART-1 plasmid (ATCC Deposit #75738) the sense strand is under the control of the T7 promoter, the antisense strand is under the SP6 promoter. It is preferable that the probe be hydrolyzed to a probe length of approximately 400-200 base pairs. Conventional methods for preparation of tissues for *in situ*, synthesis of probes and detection of signal can be found in Ausubel et. al., (eds) (1987) in "Current Protocols in Molecular Biology," John Wiley and Sons, New York, New York Chapter 14 and Vander Ploeg, M., Raap A.K. (1988) In "New Frontiers in Cytology" Goerttler, K., Feichter, GE, Witte. S. (eds) pp 13-21 Springer-Verlag, New York. The probe is then contacted with mammalian tissue sections and in situ analyses performed by conventional methods. Examples of tissues that can be used include, but are not limited to, mammalian embryos, adult mammalian tissues, such as skin, lymph nodes and retina, biopsy specimens, pathology specimens and necropsy specimens. In a preferred embodiment, MART-1 in situ probes may be used to evaluate MART-1 RNA expression in diseased tissue for invasive early melanoma to characterize radial and vertical growth phases of the melanoma lesion and assess the margins of the disease within the tissue.

In yet another embodiment of this invention all or parts thereof of the MART-1 (SEQ ID NO:1) nucleic acid sequence can be used to generate transgenic animals. Preferably the MART-1 gene is introduced into an animal or an ancestor of the animal at an embryonic stage, preferably at the one cell stage and generally not later than about the eight cell stage. There are several means by which transgenic animals carrying a MART-1 gene can be made. One method involves the use of retroviruses carrying all or part of the MART-1 sequence. The retroviruses containing the transgene are introduced into the embryonic animal by transfection. Another methods

involves directly injecting the transgene into the embryo. Yet another methods employs the embryonic stem cell method or homologous recombination method known to workers in the field. Examples of animals into which the MART-1 transgene can be introduced include but are not limited to, primates, mice, rats or other rodents. Such transgenic animals may be useful as biological models for the study of melanoma and to evaluate diagnostic or therapeutic methods for melanoma.

This invention further comprises an antibody or antibodies reactive with the MART-1 protein or peptides or modified peptides or analogs thereof having the amino acid sequence defined in Figure 1 (SEQ ID NO: 2) or a unique portion thereof. In this embodiment of the invention the antibodies are monoclonal or polyclonal in origin. MART-1 protein or peptides used to generate the antibodies may be from natural or recombinant sources or generated by chemical synthesis. Natural MART-1 proteins can be isolated from mammalian biological samples. Biological samples include, but are not limited to mammalian tissues such as fresh melanoma, skin, retina, primary or continuous cultures of mammalian cells such as melanoma cultures or cultured melanocytes. The natural MART-1 proteins may be isolated by the same methods described above for recombinant proteins. Recombinant MART-1 proteins or peptides may be produced and purified by conventional methods. Synthetic MART-1 peptides may be custom ordered or commercially made based on the predicted amino acid sequence of the present invention (Figure 1; SEQ ID:2) or synthesized by methods known to one skilled in the art (Merrifield, R.B. (1963) J. Amer. Soc. 85:2149). Examples of MART-1 peptides include, but are not limited to, are AAGIGILTV (M9-2; SEQ ID NO: 4), EAAGIGILTV (M10-3; SEQ ID NO: 17) and AAGIGILTVI (M10-4; SEQ ID NO: 18) (peptides are presented in single letter amino acid code). The most preferred peptide is AAGIGILTV

(SEQ ID NO:4).

Alternatively, peptides derived form the MART-1 protein sequence may modified to increase their immunogenicity by enhancing binding of the peptide to the MHC molecules in which the peptide is presented. Examples of such modified MART-1 peptides that may used are shown, but not limited to, the peptides in Table 14. In a preferred embodiment the MART-1 peptide that is modified to enhance its binding to MHC Class I molecules is AAGIGILTV (SEQ ID NO:4). By way of example, the modified peptides ALGIGILTV (M9-2-2L) (SEQ ID NO:50), WAGIGILTV (M9-2-1W) (SEQ ID NO:53), FAGIGILTV (M9-2-1F) (SEQ ID NO:54) and AAYIGILTV (M9-2-3Y) (SEQ ID NO:58). The peptide or modified peptide may be conjugated to a carrier molecule to enhance the antigenicity of the peptide. Examples of carrier molecules, include, but are not limited to, human albumin, bovine albumin, lipoprotein and keyhole limpet hemo-cyanin ("Basic and Clinical Immunology" (1991) Stites, D.P. and Terr A.I. (eds) Appleton and Lange, Norwalk Connecticut, San Mateo, California).

Exemplary antibody molecules for use in the detection methods of the present invention are intact immunoglobulin molecules, substantially intact immunoglobulin molecules or those portions of an immunoglobulin molecule that contain the antigen binding site, including those portions of immunoglobulin molecules known in the art as F(ab), F(ab'); $F(ab')_2$ and F(v). Polyclonal or monoclonal antibodies may be produced by methods known in the art. (Kohler and Milstein (1975) Nature 256, 495-497; Campbell "Monoclonal Antibody Technology, the Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (eds.) (1985) "Laboratory Techniques in Biochemistry and Molecular Biology," Volume 13, Elsevier Science Publishers, Amsterdam). The antibodies or antigen binding fragments may also be produced by genetic

engineering. The technology for expression of both heavy and light chain genes in E. coli is the subject of the PCT patent applications: publication number WO 901443, WO 901443 and WO 9014424 and in Huse et al. (1989) Science 246:1275-1281.

The antibodies of this invention may react with native or denatured MART-1 protein, peptides or analogs thereof, or modified peptides an analogs thereof. The specific immunoassay in which the antibodies are to be used will dictate which antibodies are desirable. Antibodies may be raised against the MART-1 protein or portions thereof or against synthetic peptides homologous to the MART-1 amino acid sequence.

In one embodiment the antibodies of this invention are used in immunoassays to detect the novel MART-1 protein in biological samples. In this method the antibodies of the present invention are contacted with a biological sample and the formation of a complex between the MART-1 antigen and antibody is detected. Immunoassays of the present invention may be radioimmunoassay, Western blot assay, immunofluorescent assay, enzyme immunoassay, chemiluminescent assay, immunohistochemical assay and the like. (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Press, New York, New York; Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York). Standard techniques known in the art for ELISA are described in Methods in Immunodiagnosis, 2nd Edition, Rose and Bigazzi, eds., John Wiley and Sons, New York 1980 and Campbell et al., Methods of Immunology, W.A. Benjamin, Inc., 1964, both of which are incorporated herein by reference. Such assays may be direct, indirect, competitive, or noncompetitive immunoassays as described in the art (In "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Pres, NY, NY; Oellirich,

M. 1984. J. Clin. Chem. Clin. Biochem. 22: 895-904) Biological samples appropriate for such detection assays include mammalian tissues, melanoma and melanocyte cell lines, skin, retina, lymph nodes, pathology specimens, necropsy specimens, and biopsy specimens. Proteins may be isolated from biological samples by conventional methods described in (Ausubel et al., (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York).

The antibodies of this invention can therefore be used in immunoassays to detect MART-1 antigen or alteration in the level of expression of the MART-1 antigen in biological samples isolated from mammals afflicted with a disease or disorder. Examples of biological samples include, but are not limited to, mammalian tissues, biopsy tissue samples, melanoma and lymph node biopsy samples, pathology and tissue samples. Examples of diseases that can be assessed by these immunoassays, include, but are not limited to, melanomas and tissues which are secondary sites for melanoma metastasis. By alteration in level of expression, we mean an increase or decrease of the MART protein or portions thereof relative to a control sample. Alteration is also meant to encompass substitution, deletion or addition mutants of the MART-1 protein. Such mutations can be determined by using the antibodies of this invention known to react with specific epitopes of the MART-1 protein and determining which epitopes are present relative to a control. The antibodies of this invention can therefore be used in an immunoassay to diagnose, assess or prognoses a mammal afflicted with the disease.

In a preferred embodiment, the MART-1 antibodies of this invention are used to assess the presence of the MART-1 antigen from a tissue biopsy of a mammal afflicted with melanoma using immunocytochemistry. Such assessment of the delineation of the MART-1 antigen in a diseased

tissue can be used to prognose the progression of the disease in a mammal afflicted with the disease. Specifically the MART-1 antibodies can be used to characterize the radial and vertical growth phases of the melanoma lesion. Conventional methods for immunohistochemistry are described in (Harlow and Lane (eds) (1988) In "Antibodies A Laboratory Manual", Cold Spinning Harbor Press, Cold Spring Harbor, New York; Ausbel et al. (eds) (1987). In Current Protocols In Molecular Biology, John Wiley and Sons (New York, New York).

In another embodiment, antibodies of this invention may be used to purify the MART-1 protein or portions thereof. Immunoaffinity chromatography can be performed by conventional methods known to one skilled in the art (Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, New York).

In another preferred embodiment rabbit antisera containing antibodies which specifically recognize the MART-1 protein is used to detect said protein in Western Blot Analysis. Such antisera is directed to all, or a part or parts of the MART-1 protein or synthetic peptides derived from the MART-1 protein sequence. Preferably a MART-1 synthetic peptide derived from the MART-1 predicted amino acid sequence is used (Figure 1; SEQ ID NO:2). Alternatively, modified MART-1 peptides may be used. The peptide is synthesized by standard methods on an automated peptide synthesizer and purified by high pressure liquid chromatography (HPLC) as described in Example 2. The purified peptide may be conjugated to a carrier as described in (M. Bodanszky (1984) "Principles of Peptide Synthesis," Springer Verlag, New York, New York). Using conventional methods, rabbits may be immunized with the MART-1 protein or peptide conjugated to carriers. Preferably about 0.1 to about 10 (mg) of antigen in

adjuvant may be used, most preferably about 1 mg of antigen in adjuvant may be used. The animal receives similar booster doses and antisera titer is assessed by ELISA assay. Satisfactory levels of antisera are obtained when the anti-peptide antibody titer reaches a plateau. This antibody can be used in the standard immunoassays described above.

T-lymphocytes recognize antigen in association with Class I or Class II MHC molecules in the form of a peptide fragment bound to an MHC molecule. The degree of peptide binding to a given MHC allele is based on amino acids at particular positions within the peptide (Parker et al. (1992) Journal of Immunology 149:3580; Kubo, et al. (1994) Journal of Immunology 52 :3913-3924; Ruppert J. et al. (1993) Cell 74:929-937; Falk et al. (1991) Nature 351:290-296, each of which is herein incorporated by reference). Therefore, another embodiment of this invention relates to peptides derived from the MART-1 protein sequence (Figure 1; SEQ ID NO:2) which have been modified to increase immunogenicity by enhancing binding of the peptide to the MHC molecule with which the peptide is associated. By way of example, modification may include substitution, deletion or addition of an amino acid in the given immunogenic peptide sequence or mutation of existing amino acids within the given immunogenic peptide sequence, or derivatization of existing amino acids within the given immunogenic peptide sequence. Any amino acid comprising the immunogenic peptide sequence may be modified in accordance with this invention. In a preferred embodiment at least one amino acid is substituted or replaced within the given immunogenic peptide sequence. Any amino acid may be used to substitute or replace a given amino acid within the immunogenic peptide sequence. Modified peptides are intended to include any immunogenic MART-1 peptide which has been modified and exhibits enhanced binding to the MHC molecule with which it associates when

presented to the T-cell.

By way of example, the HLA-A2 allele binds peptides of nine or ten amino acids. Examples of positions within the peptide that may be altered to enhance binding include, but are not limited to, the first position, the second position, the third position and the last position of the peptide. Any amino acid may be used to substitute or replace these positions within the immunogenic peptide sequence. For enhanced binding to HLA-A2 the amino acid at the second position of the peptide is preferably a hydrophobic aliphatic amino acid. Examples of amino acids that may be used at the second position include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threonine or glycine. Preferably leucine or methionine is found at the second position of the peptide. The last amino acid of the peptide (either the 9th or 10th amino acid depending on the length of the peptide) is preferably a hydrophobic aliphatic amino acid. Examples of amino acids that may be used in the last position of the peptide include, but are not limited to, valine, methionine, leucine, alanine, isoleucine, threonine or glycine. Preferably valine is found at the last position in the peptide. The amino acids at the first and third positions in the peptide may also be modified to enhance binding of the peptide to the MHC Class I molecule. The amino acids at the first and third positions in the peptide may be any amino acid. Preferably, the amino acids at the first and third positions are hydrophobic aliphatic amino acids or aromatic amino acids. Examples of amino acids that maybe used at these positions include, but are not limited to, leucine, methionine, valine, alanine, isoleucine, threonine, glycine, tryptophan, phenylalanine, tyrosine, serine, aspartic acid, or lysine. Examples of MART-1 peptides that may be modified include, but are not limited to, AAGIGILTV (SEQ ID NO: 4), EAAGIGILTV (SEQ ID NO: 17) and AAGIGILTVI (SEQ ID NO: 18)

(peptides are presented in single letter amino acid code). By way of example the immunogenic MART-1 peptide AAGIGILTV (SEQ ID NO:4) may be modified according to the following formula $X_1X_2X_3IGILTX_4$ (SEQ ID NO:122) wherein:

X_1 may be any amino acid, preferably any hydrophobic aliphatic amino acid, or aromatic amino acid. Examples of amino acids that may be used, but are not limited to, alanine, tryptophan, phenylalanine, tyrosine, lysine, isoleucine, leucine, methionine, threonine, glycine or serine.

X_2 may be any hydrophobic amino acid, preferably an aliphatic hydrophobic amino acids. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, isoleucine, valine, threonine, alanine or glycine.

X_3 may be any amino acid, preferably any hydrophobic aliphatic amino acid, or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, tryptophan, phenylalanine, tyrosine, lysine, aspartic acid, serine, alanine, glycine, isoleucine, valine, or threonine.

X_4 may be any hydropbic amino acid, preferably a hydrophobic aliphatic amino acid. Examples of amino acids that may be used include, but are not limited to, valine, leucine, isoleucine, alanine, threonine, or glycine.

Examples of modified AAGIGILTV (SEQ ID NO:4) peptide sequences that may be produced are shown but not limited to the peptides in Table 14 (Example 5).

This invention further includes analogs of these immunogenic modified peptides derived from the MART-1 amino acid sequence (Figure 1; SEQ ID NO:2) which have been modified. The term analog is intended to include any peptide which displays the functional aspects of these modified peptides. The term analog also includes conservative substitutions or chemical derivatives of

these modified peptides as described above. These modified peptides may be synthetically or recombinantly produced by conventional methods.

The recombinant or natural MART-1 protein, peptides, or analogs thereof, or modified MART-1 peptides, or analogs thereof may be used as a vaccine either prophylactically or therapeutically. When provided prophylactically the vaccine is provided in advance of any evidence of melanoma. The prophylactic administration of the MART-1 vaccine should serve to prevent or attenuate melanoma in a mammal. In a preferred embodiment mammals, preferably human, at high risk for melanoma are prophylactically treated with the vaccines of this invention. Examples of such mammals include, but are not limited to, humans with a family history of melanoma, humans with a history of atypical moles, humans with a history of FAM-M syndrome or humans afflicted with melanoma previously resected and therefore at risk for reoccurrence. When provided therapeutically, the vaccine is provided to enhance the patient's own immune response to the tumor antigen present on the melanoma or metastatic melanoma. The vaccine, which acts as an immunogen, may be a cell, cell lysate from cells transfected with a recombinant expression vector, cell lysates from cells transfected with a MART-1 recombinant expression vector, or a culture supernatant containing the expressed protein. Alternatively, the immunogen is a partially or substantially purified recombinant MART-1 protein, peptide or analog thereof or modified peptides or analogs thereof. The proteins or peptides may be conjugated with lipoprotein or administered in liposomal form or with adjuvant.

While it is possible for the immunogen to be administered in a pure or substantially pure form, it is preferable to present it as a pharmaceutical composition, formulation or preparation.

The formulations of the present invention, both for veterinary and for human use, comprise an immunogen as described above, together with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. The formulations may conveniently be presented in unit dosage form and may be prepared by any method well-known in the pharmaceutical art.

All methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired formulation.

Formulations suitable for intravenous intramuscular, subcutaneous, or intraperitoneal administration conveniently comprise sterile aqueous solutions of the active ingredient with solutions which are preferably isotonic with the blood of the recipient. Such formulations may be conveniently prepared by dissolving solid active ingredient in water containing physiologically compatible substances such as sodium chloride (e.g. 0.1-2.0M), glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering said solution sterile. These may be present in unit or multi-dose containers, for example, sealed ampoules or vials.

The formulations of the present invention may incorporate a stabilizer. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inor-

ganic acids, and organic acids which may be used either on their own or as admixtures. These stabilizers are preferably incorporated in an amount of 0.11-10,000 parts by weight per part by weight of immunogen. If two or more stabilizers are to be used, their total amount is preferably within the range specified above. These stabilizers are used in aqueous solutions at the appropriate concentration and pH. The specific osmotic pressure of such aqueous solutions is generally in the range of 0.1-3.0 osmoles, preferably in the range of 0.8-1.2. The pH of the aqueous solution is adjusted to be within the range of 5.0-9.0, preferably within the range of 6-8. In formulating the immunogen of the present invention, anti-adsorption agent may be used.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymer to complex or absorb the proteins or their derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyester, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled-release preparations is to incorporate the MART-1 protein, peptides and analogs thereof into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxy-methylcellulose or gelatin-microcapsules and

poly(methylmethacylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions.

When oral preparations are desired, the compositions may be combined with typical carriers, such as lactose, sucrose, starch, talc magnesium stearate, crystalline cellulose, methyl cellulose, carboxymethyl cellulose, glycerin, sodium alginate or gum arabic among others.

The proteins of the present invention may be supplied in the form of a kit, alone, or in the form of a pharmaceutical composition as described above.

Vaccination can be conducted by conventional methods. For example, the immunogen can be used in a suitable diluent such as saline or water, or complete or incomplete adjuvants. Further, the immunogen may or may not be bound to a carrier to make the protein immunogenic. Examples of such carrier molecules include but are not limited to bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), tetanus toxoid, and the like. The immunogen also may be coupled with lipoproteins or administered in liposomal form or with adjuvants. The immunogen can be administered by any route appropriate for antibody production such as intravenous, intraperitoneal, intramuscular, subcutaneous, and the like. The immunogen may be administered once or at periodic intervals until a significant titer of anti-MART-1 immune cells or anti-MART-1 antibody is produced. The presence of anti-MART-1 immune cells may be assessed by measuring the frequency of precursor CTL (cytoxic T-lymphocytes) against MART-1 antigen prior to and after immunization by a CTL precursor analysis assay (Coulie, P. et al., (1992) International Journal Of Cancer 50:289-297). The antibody may be detected in the serum using the immunoassay described above.

The administration of the vaccine or immunogen of the

present invention may be for either a prophylactic or therapeutic purpose. When provided prophylactically, the immunogen is provided in advance of any evidence or in advance of any symptom due to melanoma. The prophylactic administration of the immunogen serves to prevent or attenuate melanoma in a mammal. When provided therapeutically, the immunogen is provided at (or shortly after) the onset of the disease or at the onset of any symptom of the disease. The therapeutic administration of the immunogen serves to attenuate the disease.

A preferred embodiment is a vaccine prepared using recombinant MART-1 protein or peptide expression vectors. To provide a vaccine to an individual a genetic sequence which encodes for all or part of the MART-1 nucleic acid sequence is inserted into a expression vector, as described above, and introduced into the mammal to be immunized. Examples of vectors that may be used in the aforementioned vaccines include, but are not limited to, defective retroviral vectors, adenoviral vectors vaccinia viral vectors, fowl pox viral vectors, or other viral vectors (Mulligan, R.C., (1993) Science 260:926-932). The viral vectors carrying all or part of the MART-1 nucleic sequence can be introduced into a mammal either prior to any evidence of melanoma or to mediate regression of the disease in a mammal afflicted with melanoma. Examples of methods for administering the viral vector into the mammals include, but are not limited to, exposure of cells to the virus ex vivo, or injection of the retrovirus or a producer cell line of the virus into the affected tissue or intravenous administration of the virus. Alternatively the viral vector carrying all or part of the MART-1 nucleic acid sequence may be administered locally by direct injection into the melanoma lesion or topical application in a pharmaceutically acceptable carrier. The quantity of viral vector, carrying all or part of the MART-1 nucleic acid sequence, to be administered is based

on the titer of virus particles. A preferred range of the immunogen to be administered may be about 10^6 to about 10^{11} virus particles per mammal, preferably a human. After immunization the efficacy of the vaccine can be assessed by production of antibodies or immune cells that recognize the antigen, as assessed by specific lytic activity or specific cytokine production or by tumor regression. One skilled in the art would know the conventional methods to assess the aforementioned parameters. If the mammal to be immunized is already afflicted with melanoma or metastatic melanoma the vaccine can be administered in conjunction with other therapeutic treatments. Examples of other therapeutic treatments includes, but are not limited to, adoptive T cell immunotherapy, coadministration of cytokines or other therapeutic drugs for melanoma.

Alternatively all or parts thereof of a substantially or partially purified the MART-1 protein may be administered as a vaccine in a pharmaceutically acceptable carrier. Ranges of MART-1 protein that may be administered are about 0.001 to about 100 mg per patient, preferred doses are about 0.01 to about 100mg per patient. In a preferred embodiment, the MART-1 peptide AAGIGILTV (SEQ ID NO: 4) (presented in single letter code) or analogs thereof is administered therapeutically or prophylactically to a mammal in need of such treatment. Alternatively, modified MART-1 peptides, examples of which are presented in Table 14 may be used. Preferred doses may be about 0.001 mg to about 100 mg, most preferred are about 0.01 mg to about 100 mg. The peptide may be synthetically or recombinantly produced. Immunization is repeated as necessary, until a sufficient titer of anti-immunogen antibody or immune cells has been obtained.

In yet another alternative embodiment a viral vector, such as a retroviral vector, can be introduced into mammalian cells. Examples of mammalian cells into which the retroviral vector can be introduced include, but are

not limited to, primary mammalian cultures or continuous mammalian cultures, COS cells, NIH3T3, or 293 cells (ATTC #CRL 1573). The means by which the vector carrying the gene may be introduced into a cell includes, but is not limited to, microinjection, electroporation, transfection or transfection using DEAE dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (EDS) (1989) in "Molecular Cloning. A laboratory manual", Cold Spring Harbor Press Plainview, New York). The mammalian cells expressing the MART-1 antigen can be administered to mammals and serve as a vaccine or immunogen. Examples of how the cells expressing MART-1 antigens can be administered include, but is not limited to, intravenous, intraperitoneal or intralesional. In a preferred embodiment, the part of the MART-1 nucleic acid sequence corresponding to the peptide AAGIGILTV (SEQ ID NO: 4) is inserted into the MART-1 expression vector and introduced into the mammalian cells. Alternatively, a nucleic acid sequence corresponding to MART-1 peptides which have been modified to enhance their binding to MHC molecules may be used. By way of example, the nucleic acid sequences encoding the modified peptides shown in Table 14 may be inserted into an expressions vector and introduced with mammalian cells.

The vaccine formulation of the present invention comprise an immunogen that induces an immune response directed against the melanoma associated antigens such as the melanoma associated MART-1 antigen. The vaccine formulations may be evaluated first in animal models, initially rodents, and in nonhuman primates and finally in humans. The safety of the immunization procedures is determined by looking for the effect of immunization on the general health of the immunized animal (weight change, fever, appetite behavior etc.) and looking for pathological changes on autopsies. After initial testing in animals, melanoma cancer patients can be tested.

Conventional methods would be used to evaluate the immune response of the patient to determine the efficiency of the vaccine.

In yet another embodiment of this invention all, part, or parts of the MART-1 protein or MART-1 peptides or analogs thereof, or modified MART-1 peptides or analogs thereof, may be exposed to dendritic cells cultured in vitro. The cultured dendritic cells provide a means of producing T-cell dependent antigens comprised of dendritic cell modified antigen or dendritic cells pulsed with antigen, in which the antigen is processed and expressed on the antigen activated dendritic cell. The MART-1 antigen activated dendritic cells or processed dendritic cell antigens may be used as immunogens for vaccines or for the treatment of melanoma. The dendritic cells should be exposed to antigen for sufficient time to allow the antigens to be internalized and presented on the dendritic cells surface. The resulting dendritic cells or the dendritic cell process antigens can than be administered to an individual in need of therapy. Such methods are described in Steinman et al. (WO93/208185) and in Banchereau et al. (EPO Application 0563485A1) which are incorporated herein by reference.

In yet another embodiment of this invention T-cells isolated from individuals can be exposed to the MART-1 protein or portions thereof, or MART-1 peptides or analogs thereof or MART-1 modified peptides or analogs thereof in vitro and then administered to a patient in need of such treatment in a therapeutically effective amount. Examples of where T-lymphocytes can be isolated, include but are not limited to, peripheral blood cells lymphocytes (PBL), lymph nodes, or tumor infiltrating lymphocytes (TIL). Such lymphocytes can be isolated from the individual to be treated or from a donor by methods known in the art and cultured in vitro (Kawakami, Y. et al. (1989) J. Immunol. 142: 2453-3461). Lymphocytes are cultured in media such

as RPMI or RPMI 1640 or AIM V for 1-10 weeks. Viability is assessed by trypan blue dye exclusion assay. The lymphocytes are exposed to all or part of the MART-1 protein for part or all of the culture duration. In a preferred embodiment the lymphocytes are exposed to the AAGIGILTV (SEQ ID NO: 4) peptide (presented in single letter code) at a concentration of about 1 to about 10 micrograms(ug)/ml per 10^7 cells for all or part of the duration of lymphocyte culture. After being sensitized to the peptide the T-lymphocytes are administered to the mammal in need of such treatment. Alternatively, the modified MART-1 peptides shown in Table 14 may be exposed to lymphocytes. Examples of how these sensitized T-cells can be administered to the mammal include but are not limited to, intravenously, intraperitoneally or intralesionally. Parameters that may be assessed to determine the efficacy of these sensitized T-lymphocytes include, but are not limited to, production of immune cells in the mammal being treated or tumor regression. Conventional methods are used to assess these parameters. Such treatment can be given in conjunction with cytokines or gene modified cells (Rosenberg, S.A. et al. (1992) Human Gene Therapy, 3: 75-90; Rosenberg, S.A. et al. (1992) Human Gene Therapy, 3: 57-73).

In addition to use as a vaccine, the compositions can be used to prepare antibodies to MART-1 antigen, peptides or analogs thereof, or modified MART-1 peptides and analogs thereof. The antibodies can be used directly as anti-melanoma agents. To prepare antibodies, a host animal is immunized using the MART-1 protein, peptides or analogs thereof, or modified peptides or analogs thereof as the immunogen and bound to a carrier as described above for vaccines. The host serum or plasma is collected following an appropriate time interval to provide a composition comprising antibodies reactive with the immunogen. The gamma globulin fraction or the IgG

antibodies can be obtained, for example, by use of saturated ammonium sulfate or DEAE Sephadex, or other techniques known to those skilled in the art. The antibodies are substantially free of many of the adverse side effects which may be associated with other anti-cancer agents such as chemotherapy.

The antibody compositions can be made even more compatible with the host system by minimizing potential adverse immune system responses. This is accomplished by removing all or a portion of the Fc portion of a foreign species antibody or using an antibody of the same species as the host animal, for example, the use of antibodies from human/human hybridomas. Humanized antibodies (i.e., nonimmunogenic in a human) may be produced, for example, by replacing an immunogenic portion of an antibody with a corresponding, but nonimmunogenic portion (i.e., chimeric antibodies). Such chimeric antibodies may contain the reactive or antigen binding portion of an antibody from one species and the Fc portion of an antibody (nonimmunogenic) from a different species. Examples of chimeric antibodies, include but are not limited to, non-human mammal-human chimeras, rodent-human chimeras, murine-human and rat-human chimeras (Robinson et al., International Patent Application 184,187; Taniguchi M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., PCT Application WO 86/01533; Cabilly et al., 1987 Proc. Natl. Acad. Sci. USA 84:3439; Nishimura et al., 1987 Canc. Res. 47:999; Wood et al., 1985 Nature 314:446; Shaw et al., 1988 J. Natl. Cancer Inst. 80: 15553, all incorporated herein by reference).

General reviews of "humanized" chimeric antibodies are provided by Morrison S., 1985 Science 229:1202 and by Oi et al., 1986 BioTechniques 4:214.

Suitable "humanized" antibodies can be alternatively produced by CDR or CEA substitution (Jones et al., 1986

Nature 321:552; Verhoeyan et al., 1988 Science 239:1534; Biedleret al. 1988 J. Immunol. 141:4053, all incorporated herein by reference).

The antibodies or antigen binding fragments may also be produced by genetic engineering. The technology for expression of both heavy and light cain genes in E. coli is the subject the following PCT patent applications; publication number WO 901443, WO901443, and WO 9014424 and in Huse et al., 1989 Science 246:1275-1281.

The antibodies can also be used as a means of enhancing the immune response. The antibodies can be administered in amounts similar to those used for other therapeutic administrations of antibody. For example, pooled gamma globulin is administered at a range of about 1mg to about 100mg per patient. Thus, antibodies reactive with the MART-1 antigen can be passively administered alone or in conjunction with other anti-cancer therapies to a mammal afflicted with melanoma. Examples of anti-cancer therapies include, but are not limited to, chemotherapy, radiation therapy, adoptive immunotherapy therapy with TIL.

Alternatively, anti MART-1 antigen antibodies can be induced by administering anti-idiotype antibodies as immunogens. Conveniently, a purified anti-MART-1 antibody preparation prepared as described above is used to induce anti-idiotype antibody in a host animal. The composition is administered to the host animal in a suitable diluent. Following administration, usually repeated administration, the host produces anti-idiotype antibody. To eliminate an immunogenic response to the Fc region, antibodies produced by the same species as the host animal can be used or the Fc region of the administered antibodies can be removed. Following induction of anti-idiotype antibody in the host animal, serum or plasma is removed to provide an antibody composition. The composition can be purified as described above for anti-MART-1 antibodies, or by affinity chroma-

tography using anti-MART-1 antibodies bound to the affinity matrix. The anti-idiotype antibodies produced are similar in conformation to the authentic MART-1-antigen and may be used to prepare an MART-1 melanoma antigen vaccine rather than using the MART-1 protein, peptides analogs or portions thereof.

When used as a means of inducing anti-MART-1 antibodies in an animal, the manner of injecting the antibody is the same as for vaccination purposes, namely intramuscularly, intraperitoneally, subcutaneously, interlesionally, or the like in an effective concentration in a physiologically suitable diluent with or without adjuvant. One or more booster injections may be desirable.

The MART-1 derived proteins or peptides or modified peptides of this invention are also intended for use in producing antiserum designed for pre- or post-disease prophylaxis. Here the MART-1 antigen, peptides or analogs thereof, or modified MART-1 peptides or analogs thereof is formulated with a suitable adjuvant and administered by injection to human volunteers, according to known methods for producing human antisera. Antibody response to the injected proteins is monitored, during a several-week period following immunization, by periodic serum sampling to detect the presence of anti-MART-1 serum antibodies, using an immunoassay as described herein.

The antiserum from immunized individuals may be administered as a prophylactic measure for individuals who are at risk of developing melanoma. The antiserum is also useful in treating an individual afflicted with melanoma for post-disease prophylaxis.

For both <u>in vivo</u> use of antibodies to MART-1 antigen and anti-idiotype antibodies and diagnostic use, it may be preferable to use monoclonal antibodies. Monoclonal anti-MART-1 antibodies or anti-idiotype antibodies can be produced as follows. The spleen or lymphocytes from an

immunized animal are removed and immortalized or used to prepare hybridomas by methods known to those skilled in the art. (Goding, J.W. 1983. Monoclonal Antibodies: Principles and Practice, Pladermic Press, Inc., NY, NY, pp. 56-97). To produce a human-human hybridoma, a human lymphocyte donor is selected. A donor known to have a melanoma carrying the MART-1 antigen may serve as a suitable lymphocyte donor. Lymphocytes can be isolated from a peripheral blood sample or spleen cells may be used if the donor is subject to splenectomy. Epstein-Barr virus (EBV) can be used to immortalize human lymphocytes or a human fusion partner can be used to produce human-human hybridomas. Primary in vitro immunization with peptides can also be used in the generation of human monoclonal antibodies. Examples of preferred MART-1 peptides, but not limited to are, AAGIGILTV (SEQ ID NO: 4), EAAGIGILTV (SEQ ID NO: 17) and AAGIGILTVI (SEQ ID NO: 18) (peptides are presented in single letter amino acid code). Most preferably AAGIGILTV (SEQ ID NO: 4) is used as the immunogen. Alternatively, peptides derived from the MART-1 amino acid sequence and modified to enhance binding of the peptide to a MHC Class I molecule may also be used. By way of example the modified peptides shown in Table 14 may be used as the immunogen.

Antibodies secreted by the immortalized cells are screened to determine the clones that secrete antibodies of the desired specificity. For monoclonal MART-1 antigen or peptide antibodies, the antibodies must bind to MART-1 antigen or peptide. For monoclonal anti-idiotype antibodies, the antibodies must bind to anti-MART-1 antibodies. Cells producing antibodies of the desired specificity are selected.

The antibodies or chimeric antibodies described herein may also be coupled to toxin molecules radioisotopes and drugs by conventional methods (Vitetta et al. (1991) in "Biologic Therapy of Cancer" De Vita VT, Hellman

S., Rosenberg, S.A. (eds) J.B. Lippincott Co. Philadelphia; Larson, S.M. et al. (1991) in "Biological Therapy of Cancer" De Vita V.T., Hellman S., Rosenberg, S.A. (eds) J.B. Lippincott Co., Philadelphia). Examples of toxins to which the antibodies may be coupled to include, but are not limited to, ricin or diphtheria toxin. Examples of drugs or chemotherapeutic agents include, but are not limited to, cyclophosphamide or doxorubcin. Examples of radioisotopes, include, but are not limited to, ^{131}I. Antibodies covalently conjugated to the aforementioned agents can be used in cancer immunotherapy for treating melanoma.

Local administration to the afflicted site may be accomplished through means known in the art, including, but not limited to, topical application, injection, and implantation of a porous device containing cells recombinantly expressing the infusion, implantation of a porous device in which the MART-1 antibodies or chimeric antibodies, antibodies coupled to toxins, drugs or radiolabels or portions thereof are contained.

The above described antibodies and antigen binding fragments thereof may be supplied in kit form alone, or as a pharmaceutical composition for in vivo use. The antibodies may be used for therapeutic uses, diagnostic use in immunoassays or as an immunoaffinity agent to purify the MART-1 protein or peptides as described herein.

The present invention also provides a substantially purified and isolated nucleic acid sequence, designated c(complementary)DNA25 (Figures 4A and 4B; SEQ ID NO: 26) which encodes a second melanoma recognized by tumor infiltrating lymphocytes. The TIL which recognize the melanoma antigen encoded by cDNA25 are associated with in vivo tumor rejection. The TIL recognized the melanoma antigen encoded by cDNA25 in the context of HLA-A2. Comparison of the cDNA25 nucleic acid sequence (Figures 4A and 4B; SEQ ID NO: 26) with the nucleic acid sequences for

genes encoding a melanocyte-melanoma specific protein gp100 shows this sequence to be similar, but distinct, from the previously identified sequences for gp100. Previously identified sequences for gp100 include gp100 (GenBank Accession No. M32295; also designated gp95), Pmel 17 (GenBank Accession No. M77348; Kwon et al., (1991) Proc. Natl. Acad. Sciences (USA) 88:9228-9232) and ME20 (Maresh et al. (1994) DNA and Cell Biology 13:87-95).

The cDNA25 sequence provided herein (Figures 4A and 4B; SEQ ID NO 26) differs from the previously reported gp100 sequence in Genbank (Genbank Accession No. M32295) by two nucleotides, from the Pmel 17 sequence (Kwon et al. (1991) Proc. Natl. Acad. Sciences (USA) 88: 9228-9232) by three nucleotides and a twenty one base pair deletion, and from the ME20 sequence (Maresh et al. (1994) DNA and Cell Biology 13:87-95) by a single nucleotide difference. At the amino acid level, the protein encoded by cDNA25 differs from the gp100 in GenBank (GenBank Accession # M32295) by one amino acid at position 162, by a two amino acid difference at positions 162 and 274, compared to Pmel 17 and did not contain 7 amino acids that existed in Pmel 17 at positions 588-594. Therefore, cDNA25 appears to encode for a different form of the gene for gp100. The differences between the cDNA25 nucleic acid sequence (Figures 4A and 4B; SEQ ID NO: 26) and amino acid sequence (Figure 5A; SEQ ID NO: 27) and previously reported gp100 sequences may be due to polymorphisms, allelic variations, or to mutations within the tumor. Experiments with mouse tumors have shown that new antigens recognized by T-cells can result from point mutation in the coding region of the inactive gene (Boon, T (1992) Advances in Cancer Research 58:177-210).

This invention also provides immunogenic peptides derived from gp100 protein sequences provided herein or analogs thereof. (Figure 5A and Figure 7A; SEQ ID NOS: 27 and 121). These immunogenic peptides represent

antigenic portions of the gp100 protein (Figures 5A and 7A; SEQ ID NOS: 27 and 121) recognized by TIL. Examples of immunogenic peptides include, but are not limited to, LLDGTATLRL (peptide G10-4; SEQ ID NO: 33), VLYRYGSFSV (peptide G10-5; SEQ ID NO: 34), ALDGGNKHFL (peptide G10-22; SEQ ID NO: 35), VLKRCLLHL (peptide G9-19 SEQ ID NO: 36), VLPSPACQLV (peptide G10-8; SEQ ID NO: 37), SLADTNSLAV (peptide G10-9; SEQ ID NO: 38), SVSVSQLRA (peptide G9-216; SEQ ID NO:39), YLEPGPVTA (peptide G9-280; SEQ ID NO:40), LNVSLADTN (peptide G10-400; SEQ ID NO:41), KTWGQYWQV (peptide $G9_{154}$; SEQ ID NO:46; Figure 7A; amino acids 154 to 162), KTWGQYWQVL (peptide $G10_{154}$ SEQ ID NO:47; Figure 7A; amino acids 154 to 163), ITDQVPFSV (peptide $G9_{209}$, SEQ ID NO:48; Figure 7A; amino acids 209 to 217) and TITDQVPFSV (peptide $G10_{208}$; SEQ ID NO: 49; Figure 7A; amino acids 208 to 217). This invention further includes analogs of these immunogenic peptides derived from gp100 amino acid sequence (Figures 5A and 7A; SEQ ID NOS: 27 and 121). The term analog includes any peptide which displays the functional aspects of these immunogenic peptides. The term analog also includes conservative substitution or chemical derivative of the peptides as described above. These immunogenic peptides may be synthetically or recombinantly produced in the same manner or fashion as described above for MART-1.

In yet another embodiment of this invention, immunogenic peptides derived from gp100 sequences (Figure 5A and Figure 7A; SEQ ID NOS: 27 and 121) are modified to increase immunogenicity by enhancing the binding of the peptide to MHC molecule with which the peptide is

associated when presented to T-cells. By way of example, modifications may include the substitution, deletion or addition, of one or more amino acids within the immunogenic peptide sequence, or insertion of amino acids within the given immunogenic peptide sequence or derivitization of existing amino acids within the given immunogenic peptide sequence or mutation of the amino acids within the given immunogenic peptide sequence. In a preferred modification at least one amino acid is substituted or replaced in the given immunogenic peptide sequence. Any amino acid composing the given immunogenic peptide sequence may be modified in accordance with this invention. Any amino acid may be used to substitute or replace a given amino acid within the immunogenic peptide sequence. Modification may occur at any amino acid position within the immunogenic gp100 peptide. Modified gp100 peptides is intended to include any modified immunogenic gp100 peptide exhibiting enhanced binding with the MHC molecule with which it is associated when presented to the T-cell.

By way of example peptides recognized by T cells in the context of HLA-A2 are 9 to 10 amino acids in length. Preferably for enhanced binding of the peptide to HLA-A2 the second position and last position in the peptide are hydrophobic amino acids preferably aliphatic hydrophobic amino acids. The second position may be any aliphatic hydrophobic amino acid such as, but not limited to, leucine, methionine, isoleucine, valine, threonine, glycine or alanine. The last position of the peptide (position 9 or 10 depending on the peptide's length) may be any aliphatic hydrophobic amino acid, such as but not limited to valine, leucine, alanine, leucine, isoleucine, glycine, methionine, valine, or threonine.

The first and third positions of the immunogenic peptide may be substituted or replaced with any amino acid, preferably, hydrophobic aliphatic amino acids, or

aromatic amino acids. Examples of amino acids that may be used at the first or third position of the peptide include, but are not limited to, alanine, leucine, lysine, isoleucine, glycine, methionine, valine, threonine, tryptophan, phenylalanine, serine, lysine or tyrosine.

Examples of gp100 peptides that may be modified in accordance with the present embodiment include, but is not limited to LLDGTATLRL (peptide G10-4; SEQ ID NO: 33), VLYRYGSFSV (peptide G10-5; SEQ ID NO: 34), ALDGGNKHFL (peptide G10-22; SEQ ID NO: 35), VLKRCLLHL (peptide G9-19 SEQ ID NO: 36), VLPSPACQLV (peptide G10-8; SEQ ID NO: 37) SLADTNSLAV (peptide G10-9; SEQ ID NO: 38), SVSVSQLRA (peptide G9-216; SEQ ID NO:39), YLEPGPVTA (peptide G9-280; SEQ ID NO:40), LNVSLADTN (peptide G10-400; SEQ ID NO:41), KTWGQYWQV (peptide $G9_{154}$; SEQ ID NO:46; Figure 7A; amino acids 154 to 162), KTWGQYWQVL (peptide $G10_{154}$; SEQ ID NO:47; Figure 7A; amino acids 154 to 163), ITDQVPFSV (peptide $G9_{209}$, SEQ ID NO:48; Figure 7A; amino acids 209 to 217) and TITDQVPFSV (peptide $G10_{208}$; SEQ ID NO: 49; Figure 7A; amino acids 208 to 217).

By way of example modified gp100 peptides derived from the immunogenic gp100 peptide KTWGQYWQV (SEQ ID NO:46) may have the formula $X_1X_2X_3GQYWQX_4$ (SEQ ID NO:123) wherein:

X_1 may be any amino acid, preferably any hydrophobic aliphatic amino acid, or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, alanine, leucine, lysine, isoleucine, glycine, methionine, valine, threonine, tryptophan, phenylalanine,

lysine or serine, aspartic acid or tyrosine;

X_2 may be any hydrophobic amino acid, preferably any aliphatic hydrophobic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, isoleucine, alanine, threonine, glycine, or valine. Most preferably leucine, methionine or isoleucine.

X_3 may be any amino acid, preferably any hydrophobic aliphatic amino acid or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, alanine, leucine, lysine, isoleucine, glycine, methianine, valine, threonine, tryptophan, phenylalanine, serine, lysine, or tyrosine;

X_4 may be any hydrophobic amino acid, preferably an aliphatic hydrophobic amino acid. Examples of amino acids that may be used include, but are not limited to, valine, leucine, isoleucine, methionine, alanine, threonine, or glycine.

Examples of modified peptides are shown in Table 15. A preferred modified peptide is KIWGQYWQV (G9-154-2I) (SEQ ID NO:70).

Alternatively, the immunogenic gp100 ITDQVPFSV (G9-209; SEQ ID NO:48) may be modified, such modified peptides may have the general formula $X_1X_2X_3QVPFSX_4$ (SEQ ID NO:124) wherein:

X_1 may be any amino acid, preferably any hydrophobic aliphatic amino acid, or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threanine, glycine, lysine, phenylalanine, tryptophan, or tyrosine, aspartic acid or serine;

X_2 may be any hydrophobic amino acid, preferably a hydrophobic aliphatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threanine, or glycine;

X_3 may be any amino acid, preferably any hydrophobic, aliphatic amino acid or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threonine, glycine, lysine, phenylalanine, tryptophan, tyrosine, aspartic acid or serine;

X_4 may be any hydrophobic amino acid, preferably any hydrophobic aliphatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, or threonine;

Examples of modified peptides derived from ITDQVPFSV are shown in Table 16. Preferably the peptide FLDQVPFSV (peptide G9-209-1F2L) is used.

By way of example modified gp100 peptides derived from the immunogenic gp100 peptide YLEPGPVTA (G9-280; SEQ ID NO:40) may also be modified to enhance binding to MHC Class I molecules, preferably HLA-A2 or subtypes thereof.

The modified peptides may have the general formula $X_1X_2X_3$PGPVTX_4 (SEQ ID NO:125) wherein:

X_1 may be any amino acid, preferably a hydrophobic aliphatic amino acid or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threanine, glycine, lysine, phenylalanine, tryptophan, or tyrosine, aspartic acid or serine;

X_2 may be any hydrophobic amino acid, preferably an aliphatic hydrophobic amino acid. Examples of amino acids that may be used include, but are not limited to leucine, methionine, alanine, isoleucine, valine, threonine, or glycine;

X_3 may be any amino acid, preferably any hydrophobic aliphatic amino acid, or aromatic amino acid. Examples of amino acids that may be used include, but are not limited to, leucine, methionine, alanine, isoleucine, valine, threanine, glycine, lysine, phenylalanine, tryptophan,

tyrosine, aspartic acid or serine;

X_4 may be any hydrophobic amino acid preferably an aliphatic hydrophobic amino acid. Examples of amino acids that may be used include but are not limited to, leucine, methionine, alanine, isoleucine, valine, threonine, or glycine.

Examples of modified peptides derived from YLEPGPVTA (G9-280; SEQ ID NO:40) are shown in Table 17. A preferred modified peptide is YLEPGPVTV (G9-280-9V) (SEQ ID NO:104).

This invention further includes analogs of these modified peptides derived from the gp-100 sequences disclosed herein (Figure 5A; SEQ ID NO:27 and FIGURE 7A SEQ ID NO:121). The term analog is intended to include any peptide which displays the functional aspects of these modified peptides as described above. These modified peptides may be synthetically or recombinantly provided by conventional methods.

In another embodiment the immunogenic peptides derived from gp100 amino acid sequences or modified gp100 peptides as shown in Tables 15-17 or analogs thereof, may be used as a vaccine either therapeutically or prophylactically. When provided, prophylactically the vaccine is provided in advance of any evidence of melanoma. The prophylactic administration of these peptides should serve to prevent or attenuate melanoma in a mammal.

In a preferred embodiment, mammals, preferably humans, at high risk for melanoma are prophylactically treated with these vaccines. Alternatively, the vaccine may be provided therapeutically to enhance the patients own immune response to the tumor antigen prescribed on the melanoma or metastatic melanoma. The vaccine, which acts as an immunogen, may be a cell, cell lysate from cells transfected with a recombinant expression vector carrying a nucleic acid sequences encoding gp100 immunogenic peptide or a culture supernatant containing the expressed

protein. Expression vectors into which nucleic acid sequences encoding these immunogenic peptides may be introduced are the same as those described above for MART-1. Alternatively, the immunogen is a partially or substantially purified recombinant gp100 peptide or analog thereof.

While it is possible for the immunogen to be administered in a pure or substantially pure form, it is preferable to present it as pharmaceutical compositions, formulations or preparations as described above for MART-1. Vaccination can be conducted by conventional methods previously described above for MART-1.

The gp100 immunogenic peptides and nucleic acids sequences encoding them may be used in bioassays, or to generate antibodies in the same manner or fashion as described above for MART-1.

In yet another embodiment of this invention, multivalent vaccines against one or more melanoma antigens are provided. Such multivalent vaccines may comprise all or part of the MART-1 protein peptides or modified peptides or gp100 peptides or modified peptides or combinations thereof.

Previous identification of genes encoding melanoma antigens have utilized PBL isolated from melanoma patients immunized or pretreated with antigens (Van Der Bruggen et al. (1991) Science 254: 1643-1647; Brichard et al. (1993) J. Exp. Med. 178: 489-495; Traversari, C., et al. (1992) J. Exp. Med. 176: 1453-1457). A preferred strategy is to identify genes coding for tumor antigens that are recognized by TIL from tumor-bearing patients, in the absence of immunization of said patients. Such a strategy enhances the possibility that the genes identified code for antigens involved in the natural immune response against the growing cancer. Thus, this invention also provides a method of identifying genes encoding melanoma antigens utilizing cDNA expression cloning using tumors

infiltrating lymphocytes isolated (TIL) from the tumor of patients afflicted with melanoma. The method comprises the following steps: (a) isolating tumor infiltrating lymphocytes from the tumor of a mammal afflicted with melanoma; (b) introducing a melanoma cDNA library into a mammalian cell line; (c) exposing said mammalian cells to said TIL; (d) screening for expression of an antigen encoded by said cDNA in said mammalian cells recognized by said TIL; and (e) isolating said cDNA corresponding to said antigen. The tumor infiltrating lymphocytes in step (a) may be isolated from patients afflicted with melanoma including, but not limited to, the melanoma lesion, subcutaneous tissue or visceral organs. Examples of cells that may be used to prepare the cDNA library used in step (b), include, but are not limited to, fresh or cultured melanoma cells. Preferably, the cDNA library is introduced into mammalian cells not expressing melanoma antigens. If non human mammalian cells or human cells not expressing the desired HLA haplotype for recognition by the TIL are used in step (b), such cells can be cotransfected with an HLA gene as described below. Examples of cells which can be used in step (b), include but are not limited to, tumor cell lines, such as breast cancer cell line MDA 231 (ATCC # HTB26), or COS 7 cells (ATCC #CRL 1651). Examples of MHC genes which can be used include, but are not limited to, HLA-A, HLA-B, and HLA-C genes, preferably HLA-A2 and subtypes thereof (Zemmour, J. et al. (1992) Tissue Antigens 40:221-228). The appropriate MHC gene to be used is determined by the haplotype of the tumor cells which were the source for the cDNA library. Standard methods can be used to determine the haplotype recognized by the TIL isolated (ASHI Laboratory Manual (2nd Edition 1990). Examples of how to evaluate recognition of the cells containing the cDNA clone expressing an antigen recognized by the TIL includes, but is not limited to, γ-interferon assays, TNF

secretion (Van de Bruggen et al., (1991) Science 254:1643-1647) or lysis of cells transfected with cDNA encoding for the recognized antigen. Such assays are performed by conventional methods known to one skilled in the art. Melanoma antigens can be isolated by or rescued by PCR using primer specific to flanking site of vector containing the cDNA. Examples of how to isolate the cDNA corresponding to the antigen recognized by the TIL include, but are not limited to, PCR.

Once the genes or nucleic acid sequences encoding melanoma antigens are identified, the next step is to determine the antigenic portion or epitope of the protein encoded by these genes. Therefore, in yet another embodiment of this invention, a method is provided for assessing the immunogenicity of peptides derived from the predicted amino acid sequences of the MART-1 protein (Figure 1; SEQ ID NO: 2) or gp100 protein (Figure 5A and Figure 7A; SEQ ID NO: 27 and SEQ ID NO: 121). The method comprises the steps of: (a) preparing a plurality of peptides based on the MART-1 (Figure 1; SEQ ID NO: 2) or a gp100 (Figure 5A and Figure 7A; SEQ ID NO: 27 and SEQ ID NO: 121) amino acid sequence; (b) incubating at least one of said peptides with a mammalian cell line; (c) exposing said mammalian cells incubated with said peptide to tumor infiltrating lymphocytes (TIL); and (d) screening for recognition of TIL with said cells incubated with said peptide. It is preferred that peptides of about 25 to 5 amino acids be used, more preferably 20 to 10 amino acids and most preferably 9-10 amino acids. Examples of cells that may be used in step (b) include, but are not limited to, T2 cells, (Cerundolo, V. et al. (1990) Nature, 345: 449-452) or EBV transformed B cell lines (Topalian et al. (1989) J. Immunol. 142: 3714-3725). Examples of how to assess recognition of the cells incubated with peptide include but is not limited to, ^{51}CR release cytotoxicity assay (Cerundolo, V. et al. (1990) Nature 345:449-452.) or

lymphokine assays such as γ-IFN or TNF secretion. (Schwartzentruber, D. et al., (1991) J. of Immunology 146:3674-3681).

T cells recognize antigen complexed with MHC Class 1 molecules. The MHC locus in all mammalian species contains numerous genes and is highly polymorphic. Different MHC molecules or haplotypes types bind different antigens. In humans the HLA complex contains the HLA-A, HLA-B and HLA-C gene loci which encode class I molecules. Lymphocytes will recognize tumor antigens on the context of HLA Class 1 molecule. If the cells containing the recombinant MART-1 expression vector are to be screened by the TIL but are not human cells, such as COS cells, or do not express a desired haplotype an expression vector containing an MHC Class 1 gene may also be introduced into the cells. (See Example 1) This, represents yet another alternative embodiment of the invention. Cells expressing MART-1 antigens and HLA antigens can by screened with TIL to detect the presence of tumor antigens in the context of a specific MHC Class 1 restriction type. The appropriate haplotype is determined by the haplotype of the tumor from which the library is derived. Examples of MHC Class I genes that may be used include, but are not limited to, HLA-A, HLA-B and HLA-C genes. Examples of preferred MHC specificities or restriction types include, but is not limited to HLA-A1, HLA-A2, such as the HLA-A2.1 subtype, or HLA-A24 (Zemmour, J. et al. (1992) Tissue Antigens 40:221-228). Most preferred is the HLA-A2.1 gene.

Veterinary uses are also intended to be encompassed by the compositions and therapeutic applications described herein.

All books, articles, and patents referenced herein are incorporated by reference in their entirety. The following examples illustrate various aspects of the invention and in no way intended to limit the scope thereof.

Cloning of a Gene Coding for a Shared Human Melanoma Antigen Recognized by Autologous T Cells Infiltrating Into Tumors

Example 1

Generation of cytotoxic T lymphocytes(CTL) and culture of cell lines

CTL were generated from excised tumor specimens by culturing a suspension of cells with 6000IU/ml of IL-2 (Cetus-Oncology Division, Chiron Corp. Emeryville, CA) for 30-70 days as described in Kawakami, Y., et al. (1988) J. Exp. Med. 168:2183-2191. TIL501 and TIL1235 were predominantly $CD8^+$ and were derived from the tumor specimens of patients with advanced metastatic melanoma. The $CD8^+$ T cell clone, TIL501.A42, was established by limiting dilution methods and cultured with 120 IU/ml of IL-2 plus irradiated (once a week for 4 to 6 times) autologous tumor cells.

Melanoma cell lines, 397mel, 501mel, 526mel, 537mel, 624mel, 888mel, 952mel, and Epstein-Barr virus (EBV) transformed B cell lines, 501EBVB, 836EBVB were established in our laboratory and cultured in RPMI1640 (GIBCO/Lifetechnologies, Grand Island N.Y.) medium containing 10% fetal calf serum (FCS) (Biofluids, Rockville MD). (Topalian et al., (1989) J. Immunol. 142: 3714-3725) Normal cultured melanocytes, NHEM483, NHEM493, NHEM527, NHEM529, NHEM530, NHEM533, NHEM616 and NHEM680 were purchased from Clonetics, San Diego, CA, FM725, FM801, FM902 were provided by M. Herlyn, Wistar Institute, Philadelphia PA, HA002 was provided by R. Halaban, Yale university, New Haven, CT and cultured in melanocyte growth medium (MGM, Clonetics). Melanoma cell lines, C32, RPMI7951, WM115, A375, HS695T, Malme3M, colon cancer cell lines, Collo, SW480, WiDr, breast cancer cell lines, MDA231, MCF7, HS578, ZR75, neuroblastoma cell line, SK-N-SH, glioma cell lines, U138MG, HS683, H4, sarcoma cell line 143B, embryonal kidney cell line 293 transformed with

adenovirus type 5 were purchased from ATCC, Rockville, MD. Renal cancer cell lines, UOK108 and UOK117 were provided M. Linehan NIH, Bethesda, MD. The small cell lung cancer cell line, H1092 was provided by J.D.Minna, University Texas Southwestern, Dallas, TX. Ewing's sarcoma cell lines, TC71, RD-ES, 6647 were provided by M. Tsokos, NIH, Bethesda, MD. The neuroblastoma cell line, SK-N-AS was provided by O.M. El Badry, NIH, Bethesda, MD. The plasmacytoma cell line, HMY-C1R and the M1 fibroblast cell line were provided by W.Biddison, NIH, Bethesda, MD. Kidney epithelial cells, KAM, WLC were provided by D.J.Hazen-Martin and D.A.Sens, Medical University of South Carolina, Charleston, SC. The monkey kidney cell line, COS7, was provided by W. Leonard, NIH, Bethesda, MD.

Cytotoxicity assay

^{51}Cr release assays were performed as described in Kawakami, Y., et al., (1988) J. Exp. Med. 168:2183-2191. Briefly, 5000 target cells labeled with ^{51}Cr were mixed with varying numbers of effector cells and incubated for 5 hours(h). Then supernatants were collected, radioactivity was measured and the percent specific lysis was calculated.

IFN-γ release assay

Fifty to one hundred thousand responder cells and $4x10^4$-10^5 stimulator cells were mixed in 300ul of AIM-V medium containing 120IU/ml IL-2 per well in a 96 flat-well microplate. After incubation for 20h, 100ul of supernatants were collected and added to an enzyme-linked immunosorbent assay (ELISA) plate (Immunoplate MaxiSorp, Nunc, Denmark) coated with anti-human IFN-γ monoclonal antibody (mAb) (Biosource, Camerillo, CA). After overnight incubation at 4°C, the plates were washed 3 times and 100ul of a 1:2000 dilution of rabbit anti-human IFN-γ polyclonal antibody (Ab)(Biosource, Camerillo, CA) was added and incubated at 37°C for 2 h. Plates were washed 3 times, and 100ul of a 1:2000 dilution of

alkaline phosphatase labelled goat anti-rabbit IgG polyclonal antibody (Ab) (Boehringer Mannheim, Indianapolis, IN) was added. After a 1 h incubation at 37° C, 100ul of 4mg/ml p-Nitrophenyl phosphate (Sigma, St Louis, MO) was added, incubated for 10-20 min at room temperature in the dark, and 25ul of 1N NaOH was added to stop the reaction. Optical density was measured at 405nm wave length and the concentration of IFN-γ was calculated in comparison to recombinant IFN-γ standards (Biogen, Cambridge, MA) measured in the same assay.

cDNA expression cloning

A cDNA library was constructed from poly A RNA from the HLA-A2$^+$ melanoma cell line, 501mel as described in (Miki, T., et al., (1989) Gene; 83:137-146 Miki et al. (1991) Proc. Nat. Acad. Sci. USA 88:5167-5171). Briefly, first strand cDNA was synthesized with a linker primer GGACAGGCCGAGGCGGCC$(T)_{40}$ (SEQ ID NO:42) followed by second strand cDNA synthesis. After treatment with T4 DNA ligase, an SfiI adaptor consisting of two oligonucleotides, CCAATCGCGACC (SEQ ID NO:43) and GGTCGCGATTGGTAA (SEQ ID NO:44) was ligated to the end of the cDNA. The cDNA was digested with SfiI and the digested fragment was isolated by passing through a spun column. The cDNA was then mixed with bacteriophage λpCEV27 (Miki, T. et al., (1991) Proc. Natl. Acad. Science (USA) 88: 5167-5771) vector arms prepared by SfiI digestion and in vitro packaging was performed.

To screen for melanoma antigens, 10ug of the amplified cDNA library containing approximately 10^7 clones was transfected into the HLA-A2$^+$ antigen non-expressing cell lines, MDA231 clone 7 and A375 clone 1-4 using a modified calcium-phosphate method (Mammalian Transfection Kit, Stratagene). After G418 (BRL, Gaithersburg, MD) selection, individual colonies were isolated and cultured in 96 well microplates and replica plates were made. A mixture of $5x10^4$ TIL1200 and $5x10^4$ TIL1235 was added to the

wells of the microplates containing the growing transfectants that were near confluence and incubated for 20 h. Supernatants were collected and IFN-γ was measured by ELISA.

Polymerase chain reaction(PCR) was performed to rescue the transfected genes from the genomic DNA of positive transfectants using SP6 and T7 primers which flank the inserted genes. The amplified products were cloned in the pCRII vector (Invitrogen, San Diego, CA). For cDNA clones 22 and 23, a Hind III and XhoI fragment containing the full length cDNA was subcloned into the expression vector pcDNA3 (Invitrogen, San Diego, Ca).

To test whether the cloned cDNAs encode tumor antigens, the pcDNA3 containing the cloned genes were transiently transfected into the COS7 cell line by the DEAE dextran method (Seed, B. and Aruffo, A. (1987) Proc. Natl. Acad. Sci. (USA) 84:3365-3369). Briefly, $3x10^5$ cells per well in 6 well plates were incubated at 37° C for 4 hours (h) in 0.75 ml DMEM containing 100ug of DEAE dextran (Sigma), 0.1mM chloroquine and 1ug of the pcDNA3 containing the cloned genes and/or the pcDNA-HLA-A2.1 (Zemmour, J. et al. (1992) Tissue Antigens 40: 221-228). After medium was removed, 10% DMSO solution in HBSS buffer was added and incubated for 2 min. The cells were washed once with PBS and incubated in 7.5% FCS DMEM for 2 days. The 293 cell line was transiently transfected using lipofectamine (BRL, Gaithersburg, MD) according to the manufacturer's recommendation. After incubation, the ability of the transfected COS7 or 293 cells to mediate IFN-γ release from TIL was assessed. The expression of the HLA-A2 gene was tested by flow cytometry. Stable transfectants were made by the calcium-phosphate method and individual colonies and pooled transfectants were tested for reactivity to TIL by cytotoxicity and IFN-γ release assays.

DNA sequencing of the cloned genes was performed by

dideoxy chain termination method with dGTP and 7-deaza-dGTP. DNA and protein sequences were analyzed by the GCG program with GeneBank, and EMBL Data Library nucleotide databases and SWISS-PROT, PIR, GenPept, Brookhaven Protein Data Bank protein databases.

Northern blot analysis

Total RNA was isolated by the guanidine-isothiocyanate-cesium chloride centrifugation method. (Chirgwin, J.M. et al. (1979) Biochemistry 18: 5294). Total RNA from normal tissue was purchased from Clontech, (Palo Alto, CA). Ten to twenty micrograms of total RNA was subjected to electrophoresis in a 1% agarose formaldehyde gel and transferred to a nylon membrane (Duralon-UV membranes, Stratagene, La Jolla, CA) The Sal I digested fragment containing the full length cDNA from clone 22 and the ß-actin cDNA (Clontech) were labeled by random priming and used as a probe. Hybridization with the probe was performed according to the QuikHyb protocol (Stratagene) at 68 C for 2-16 h. Membranes were washed two times with 2XSSC/0.1%SDS at 60 C for 15 minutes (min) and once with 0.1XSSC at 60 C at for 30 min and then autoradiography was performed.

Characterization of cultured TIL from melanoma patients

Multiple TIL lines were established from HLA-A2$^+$ melanoma patients and tested for lysis of melanoma cell lines from HLA-A2$^+$ and HLA-A2$^-$ patients. HLA typing of patients is performed by conventional HLA typing techniques. HLA-A2 was selected because it is the most frequently expressed class I MHC antigen (about 50% of individuals) and has been shown to be a dominant restriction element for the recognition of melanoma antigens (Crowley, N.J., et al. (1991), J. Immunol. 146:1692-1694). TIL501, TIL1235 and TIL1200 exhibited specific recognition of shared melanoma antigens in an HLA-A2 restricted fashion. TIL501.A42 was a T cell clone established from TIL501 by limiting dilution. These TIL

caused lysis or released cytokines including IFN-γ, TNFα and GM-CSF when cocultured with a variety of HLA-A2$^+$ melanoma or melanocyte cell lines but not HLA-A2$^-$ melanoma lines or HLA-A2$^+$ non-melanoma cell lines including the breast cancer cell line, MDA 231. Two representative experiments are shown in Table 1. Thus, these CTL seemed to recognize a non-mutated peptide derived from a melanocyte lineage specific antigen.

Cloning of cDNA coding for melanoma antigens recognized by T cells

A cDNA library from the HLA-A2$^+$ 501mel melanoma cell line was transfected into two highly transfectable HLA-A2.1$^+$ cancer cell lines, MDA231 and A375. These cell lines were not lysed by melanoma specific TIL but were lysed by HLA-A2 restricted influenza M1 specific CTL after incubation with the $M1_{58\text{-}66}$ peptide (GILGFVFTL; single letter code (SEQ ID NO:45) derived the influenze matrix protein or infection with a recombinant vaccinia virus containing the M1 gene (data not shown). Thus, these cell lines exhibited normal antigen processing and presenting ability but were not lysed by these melanoma-specific TIL because of the lack of expression of the relevant melanoma antigens. After selection with G418, approximately 6700 transfected clones from each cell line were isolated and grown in microplates. Using the IFN-γ release assay, 21 MDA231 and 27 A375 positive clones were isolated and rescreened. Of these clones, eight MDA231 and seven A375 clones were positive in a second screening assay.

In order to rescue the integrated genes, PCR using genomic DNA from these positive transfectants was performed with SP6 and T7 primers flanking the insert genes. Eight genes that were amplified from the seven transfectants which showed 1 to 2 sharp bands, including a 1.6Kb band from MDA-22 and MDA-23 transfectants, were subcloned into the pCRII cloning vector, and then further cloned into the pcDNA3 eukaryotic expression vector. The

1.6Kb band detected by Northern blot analysis with the cDNA 22 probe suggested that this fragment was a full length cDNA.

Transient transfection of the expression vector pcDNA3 containing the cDNA from clones 22 or 23 into either COS7 or 293 cells along with the HLA-A2.1 gene conferred reactivity to TIL1235 and TIL501.A42 as demonstrated by the specific release of IFN-γ (Table 2, Experiments 1 and 2). Stable transfection of these cDNA fragments into MDA 231 or A375mel cell lines also conferred reactivity to TIL1235 and TIL501.42 (Table 2, Experiment 3). TIL501.A42 could lyse MDA231 stably transfected with cDNA 22 (data not shown). These results indicated that these cDNAs encode a melanoma antigen recognized by HLA-A2 restricted TIL from melanoma patients. Transfection of another clone, MDA-25 stimulated the release of interferon-γ only from TIL 1200.

Table 1. Specificity of TIL 501.A42 and TIL 1235 for Melanomas and Melanocytes

A. Lytic Specificity*

Target	HLA-A2	TIL501.A42	TIL1235	LAK+
			% Lysis	
501mel	+	54	51	78
526mel	+	25	33	74
624mel	+	23	27	75
952mel	+	10	11	75
Malme3M	+	36	41	70
C32	+	17	23	82
RPMI7951	+	1	6	67
WM115	+	-2	3	68
HS695T	+	1	2	87
397mel	-	-1	0	70
MDA231	+	0	3	94

B. Cytokine Release Specificity**

Stimulator Cells	HLA-A2	TIL501.A42	TIL1235	TIL586++
			pg IFN-	γ/ml
501mel	+	647	219	<50
586mel	-	<50	<50	1034
NHEM493	+	1835	850	<50
NHEM527	+	1638	749	<50
NHEM530	+	1224	2532	<50
NHEM533	+	300	251	<50
NHEM616	+	635	423	<50
FM725	+	5975	1471	<50
FM801	+	1375	893	62
NHEM483	-	<50	<50	<50
NHEM680	-	<50	<50	548
HA002	-	<50	<50	<50

TIL 501.A42 and TIL1235 lysed most HLA-A2 melanoma cell lines and secreted IFN-γ when cultured with HLA-A2 melanomas and melanocytes.

*51Cr release assay was performed at E:T = 20:1 for TIL501.A42, at 40:1 for TIL1235. All targets were melanoma cell lines except for MDA231 which was a breast cancer cell line.

**IFN-γ in the supernatant was measured after TIL and stimulator cells were coincubated for 20 hr. 501mel and 586mel are melanoma cell lines. All others were normal melanocyte cell lines.

+LAK: Lymphokine activated killer cells.

++TIL586 is class I MHC restricted melanoma specific TIL, not restricted by HLA-A2.

Characterization of this cDNA revealed it to be similar, but distinct, from a previously described melanoma antigen gp100 recognized by monoclonal antibody HMB45. This clone is described in more detail in Example 3.

The cDNA sequence of clones 22 and 23 were identical except at a single base that was believed to be a change introduced by PCR. Two other independently amplified fragments were also sequenced to clarify this region and the consensus sequence is shown in Figure 1. The longest open reading frame in this gene consists of 354 bases corresponding to a 118 amino acid protein of 13kd. This sequence did not show significant similarity to any complete nucleotide or protein sequences in established databases. Amino acids 27-47 consist of a hydrophobic region that may contain the HLA-A2 binding peptides (Falk, K., et al. (1991), Nature 351:290-296; Hunt, D.F., et al. (1992), Science 255:1261-1263; Ruppert, J., et al. (1993), Cell 74:929-937; Nijman, H.W., et al. (1993), Eur. J. Immunol. 23:1215-1219). The antigen encoded by the cDNA 22 and 23, was designated the MART-1 antigen (Melanoma Antigen Recognized by T cells-1). Of the ten HLA-A2 restricted TIL lines generated nine recognized MART-1, and four recognized a form of gp100 isolated and described herein (see Example 3) and none appeared to recognize MAGE-1 (Zakut, R., et al. (1993), Cancer Res 53:5-8.; data not shown).

Table 2. Interferon Gamma Secretion by TIL501.A42 and TIL 1235 When Cultured with HLA-A2$^+$ Cell Lines Transfected with the Gene 22 or 23

Stimulator Cell	Transfected Gene	HLA-A2	TIL501.A42	TIL1235
			pg/ml	
Exp. 1				
501mel	none	+	1009*	1076
397mel	none	-	<50	<50
COS7	none	-	<50	<50
COS7	HLA-A2.1	+	<50	<50
COS7	22	-	<50	<50
COS7	HLA-A2.1+22	+	771	1049
Exp. 2				
501mel	none	+	ND$^+$	1051
397mel	none	-	ND	<50
293	HLA-A2.1	+	ND	<50
293	22	-	ND	<50
293	HLA-A2.1+22	+	ND	255
Exp. 3				
501mel	none	+	1073	1056
397mel	none	-	<50	<50
MDA231	none	+	<50	<50
MDA231	23	+	674	725
A375	none	+	<50	<50
A375	23	+	264	131

IFN-γ in the supernatant was measured after TIL were coincubated for 20 hr with COS7 or 293 cell lines transiently transfected with the pcDNA3 containing the HLA-A2.1 and/or cDNA 22 by the DEAE-dextran method (Exp. 1 & 2), or with the A375 or MDA231 cell lines stably transfected with cDNA 23 (Exp. 3). IFN-γ was secreted only when TIL were incubated with HLA-A2$^+$ cell lines transfected with the cDNA 22 or 23.

*IFN-γ secreted by TIL alone without stimulator (<50 pg/ml) was subtracted.

$^+$Not done

Table 3. Expression of the MART-1 Gene in a Variety of Tissue and Cell Lines

Melanoma		Normal fresh tissue		Colon cancer	
				Collo	-
HLA-A2+		Brain	-	SW480	-
		Retina	+	WiDr	-
501mel	+	Adrenal gland	-		
526mel	+	Lung	-	Breast cancer	
624mel	+	Liver	-	MDA231	-
Malme3M	+	Kidney	-	MCF7	-
952mel	+	Spleen	-	HS578	-
697mel	+	Thymus	-	ZR75	-
C32	+	Testis	-		
RPMI7951	-	Fetal liver	-	Neuroblastoma	
WM115	-			SK-N-AS	-
A375	-	Non-melanoma cell lines		SK-N-SH	-
HLA-A2-		T-cell		Ewing sarcoma	
		TILA	-	TC75	-
397mel	+	TILB	-	RD-ES	-
888mel	+			6647	
537mel	+	B-cell			
586mel	+	Daudi	-	Sarcoma	
		HMY-C1R	-	143B	-
Melanocyte		501EBVB	-		
		836EBVB	-	Glioma	
NHEM483	+			U138MG	-
NHEM493	+	Fibroblast		HS683	-
NHEM529	+	M1 fibroblast	-		
NHEM530	+			Renal cell cancer	
FM902	+	Kidney epithelial cell		UOK108	-
FM906	+	KAM	-	UOK117	-
HA002	+	WLC	-		
				Small cell lung cancer	
				H1092	-

Northern blot analysis with 10-20 ug of total RNA was probed with the full length cDNA of the gene 22. The RNA from most melanomas, all melanocyte cell lines tested and retina were positive.

Expression of MART-1

Northern blot analysis of a variety of cell lines including melanoma, melanocyte and nonmelanoma cancer cell lines and normal tissues was performed to evaluate the expression of the gene coding for MART-1 (Table 3). Seven of ten HLA-A2$^+$ melanoma cell lines, all four HLA-A2$^-$ melanoma cell lines, and all seven melanocyte cell lines tested were positive for MART-1 RNA expression. In this Northern analysis, all HLA-A2$^+$ melanoma cell lines recently established in our laboratory expressed MART-1 RNA. There was a perfect correlation between MART-1 expression and lysis by TIL501.A42 in the 10 HLA-A2$^+$ melanoma lines shown in Table 3. TIL 501.A42 which recognized the MART-1 Ag lysed 13 of 17 (76%) HLA-A2$^+$ melanoma cell lines tested (data not shown). Of ten normal human tissues examined for mRNA expression by Northern blot analysis only retina was positive. No positivity was seen in any cell lines from T cells, B cells, kidney epithelial cells or fibroblasts or in 19 nonmelanoma tumors. It thus appears that MART-1 is a previously undescribed antigen expressed on melanocyte lineage cells from skin and retina that is also expressed on melanoma cells.

Studies using a panel of T cell clones and immunoselected melanoma clones (Knuth, A., et al. (1989), Proc. Natl. Acad. Sci. (USA) 86:2804-2808; Wolfel, T., et al. (1987), J. Exp. Med. 170:797-810), as well as studies analyzing HPLC fractionated peptides from melanoma cells (Slingluff, C.L., et al. (1993), J. Immunol. 150:2955-2963; Storkus, W.J., et al. (1993), J. Immunol. 151:3719-3727) suggest that multiple antigenic peptides that can provoke an immune response exist on melanomas. By cDNA cloning, two genes encoding melanoma antigens have been identified; MART-1 (Figure 1; SEQ ID NO. 1) and a gp100 gene (see Example 3; Figures 4A and 4B; SEQ ID NO. 26). MART-1 and the form of gp100 identified herein (Figures 4

& 5A; SEQ ID NOS: 26 and 27), both are recognized by HLA-A2.1 restricted TIL. The MART-1 antigen is a 118 amino acid protein of approximately 13 kd. Neither the gene nor the amino acid sequence for MART-1 have been previously described.

MART-1 RNA was expressed in 11 of 14 HLA-A2.1 positive or negative melanoma lines, and 7 of 7 melanocyte lines. With the exception of retinal tissue no MART-1 expression was found on any normal tissue tested, T-cell lines, B-cell lines, kidney epithelial lines, a fibroblast line or 19 tumor cell lines from cancers of the colon, breast, brain, kidney, lung or bone.

Another melanoma antigen, MAGE-1, has been described that is recognized by T cells derived from peripheral blood lymphocytes following repetitive in vivo or in vitro immunization (Van Der Bruggen, et al. (1991), Science 254:1643-1647).

The identification of genes associated with melanoma tumor antigens opens new possibilities for active specific immunization approaches to the immunotherapy of patients with cancer based on the introduction of these genes into viral or bacterial vector systems. The possibility exists that immune reactions induced against melanocyte-melanoma lineage antigens such as MART-1 may be generated against normal cells. Vitiligo, probably resulting from anti-melanocyte immune reactions, has been reported to be associated with a favorable prognosis in patients with melanoma (Nordlund, J.J., et al. (1983), J. Am. Acad. Dermatol. 9:689-695); Bystryn, J-C, et al. (1987), Arch. Dermatol. 123:1053-1055), and has also been reported in patients responding to chemoimmunotherapy (Richards, J.M., et al. (1992), J. Clin. Oncol. 10:1338-1343). TIL with anti melanocyte-melanoma reactivities have been administered to patients with advanced melanoma (Rosenberg, S.A., et al. (1988), N Engl J Med 319:1676-1680; Rosenberg S.A., J. Clin. Oncol. 10:180-199) and

although sporadic vitiligo has been seen in these patients, no adverse ophthalmologic effects related to the possible expression of these melanocyte antigens on retinal cells has been observed.

Because HLA-A2 is present in about 50% of individuals and the HLA-A2 restricted MART-1 antigen also appears to be widely expressed on melanomas, immunization with the MART-1 antigen may be particularly useful for the development of active immunotherapies.

Example 2

Characterization Of Immunogenic Epitopes of MART-1

Generation of melanoma specific CTL lines and a clone from TIL

Melanoma specific CTL lines were generated by culturing a single cell suspension made from metastatic melanoma with 6000U/ml of IL2 (Cetus-Oncology Division, Chiron Corp. Emeryville, CA) as previously reported (Kawakami, Y. et al., (1988) *J. Exp. Med. 168:2183*) A T-cell clone, A42 was established by limiting dilution methods from patient, 501.

Assessment of antigen recognition by CTL

^{51}Cr release cytotoxicity assays and cytokine release assays using ELISA to measure IFN-γ, GM-CSF and TNF-α were performed to analyze the reactivity of TIL as described in Kawakami, Y. et al. (1988), J. Exp. Med. 168:218 (see Example 1). Melanoma cell lines were established in the laboratory. For analysis of the recognition of known antigens by TIL, the COS7 cell line transfected with cDNAs encoding either, MART-1, gp100, or tyrosinase related protein (gp75) (Cohen, T. et al., (1990) Nucleic Acids Research 18:2807) along with HLA-A2.1 cDNA were incubated with TIL for 20h and the amount of IFN-γ secreted into the supernatant was measured by ELISA as described in Example

1. The cDNA encoding MART-1 (see Example 1) or gp100 (see Example 3) in plasmid pcDNA3 (Invitrogen, San Diego, CA) was cloned from a 501mel melanoma cDNA library by screening with TIL1235 or TIL1200, respectively (see Example 1). The cDNA encoding tyrosinase related protein(gp75) in pCEV27 plasmid was isolated from 501mel melanoma cDNA library using a probe generated by PCR based on the reported sequence of gp75 (Cohen et al. (1980) Nucleic Acids Research 18:2807).

Peptide synthesis and identification of antigenic peptides

Peptides were synthesized by a solid phase method using a Gilson AMS 422 multiple peptide synthesizer. The peptides were purified by HPLC on a Vydac C-4 column with 0.05% TFA/water-acetonitrile. To identify the antigenic peptides, TIL lysis of T2 cell lines preincubated for 2h with each peptide was measured using a ^{51}Cr release cytotoxicity assay.

HLA-A2 Restricted Melanoma Specific TIL

HLA-A2 restricted melanoma specific CTL lines and a clone, A42, were established from lymphocytes infiltrating into tumors of 10 melanoma patients. These TIL recognized autologous and most allogeneic fresh or cultured melanoma cells expressing HLA-A2, but did not recognize HLA-A2$^-$ melanomas or HLA-A2$^+$ non-melanoma cell lines (Kawakami et al. (1992) J. Immunol 148:638). They also recognized HLA-A2$^+$ normal cultured melanocytes derived from neonatal skin (see Example 1 and Kawakami, Y. et al. (1993), J. Immunotherapy 14:88). Thus, these TIL recognized non-mutated self-peptides derived from proteins expressed in melanoma and melanocytes in association with HLA-A2.

Recognition of Additional melanoma proteins by TIL

To evaluate the frequency of recognition of 4 isolated melanoma proteins including MART-1, a form of

gp100 (Figure 5A; SEQ ID NO: 26, see Example 3), and tyrosinase related protein(gp75), the reactivity of TIL to COS7 was tested on cell lines transfected with cDNAs encoding these 3 proteins with or without the cDNA encoding HLA-A2.1. One of several experiments with 9 TIL is shown in Table 4. Eight of the nine HLA-A2 restricted melanoma specific TIL secreted IFN-γ when incubated with COS7 cotransfected with MART-1 and HLA-A2.1. Only TIL1200 which is a relatively oligoclonal CTL line (Shilyansky, J. et al., (1994) Proc. Natl. Acad. Sci. (USA) 91:2829) did not respond to this COS transfectant. Four TIL (620, 660, 1143, 1200) recognized gp100 when transfected along with HLA-A2.1. TIL1200 secreted large amounts of IFN-γ compared to TIL620, 660, and 1143, suggesting that only a small subset of T-cells in these latter 3 TIL lines recognized gp100. None of these TIL recognized gp75 using this assay. Thus, MART-1 is a common melanoma antigen recognized by most HLA-A2 restricted TIL derived from melanoma patients.

Identification of MART-1 epitopes for TIL.

To identify the MART-1 epitopes for these TIL, 23 peptides were selected based on the known peptide binding motifs to HLA-A2.1 (Falk, K. et al., (1993) Nature, 351:290; Hunt, D. F. et al. (1992), Science, 255:1261; Ruppert, J. et al., (1993) Cell 74:929), synthesized (>90% purity) and screened by testing lysis of the HLA-A2.1$^+$ T2 cell line by TIL after incubation of the T2 line with each peptide (Table 5). The T2 cells (Cerundolo, V. et al.;

Table 4. Recognition of melanoma antigens by HLA-A2 restricted melanoma specific TIL

Stimulator Cell line	Transfected cDNA	HLA-A2	TIL 501	TIL 620	TIL 660	TIL 1074	TIL 1088	TIL 1128	TIL 1143	TIL 1200	TIL 1235
			IFN-γ secretion(pg/ml)								
501mel	none	+	93	720	530	670	491	272	354	736	750
397mel	none	-	0	0	0	0	0	0	0	0	0
COS7	none	-	0	0	0	0	0	0	14	0	0
COS7	HLA-A2.1	+	0	0	0	0	0	0	0	0	0
COS7	MART-1	-	0	0	0	0	0	0	12	0	0
COS7	gp100	-	0	0	0	0	0	0	14	0	0
COS7	gp75	-	0	0	0	0	0	0	0	0	0
COS7	HLA-A2.1+MART-1	+	270	196	131	625	328	52	184	0	743
COS7	HLA-A2.1+gp100	+	0	89	17	0	0	0	41	391	8
COS7	HLA-A2.1+gp75	+	0	0	0	11	0	0	7	0	0

IFN-γ was measured in the supernatants after HLA-A2 restricted melanoma specific TIL were coincubated with COS7 cells cotransfected with cDNAs encoding proteins expressed in melanoma with or without HLA-A2.1 cDNA. All TIL except TIL1200 secreted IFN-γ when cultured with COS7 cotransfected with cDNAs encoding MART-1 and HLA-A2.1. TIL620, 660, 1143 and 1200 secreted IFN-γ when cultured with COS7 cotransfected with the cDNAs encoding gp100 and HLA-A2.1.

(1990) Nature 345: 449-452) cell line was lysed well by all 4 HLA-A2 restricted melanoma specific TIL tested when preincubated with either peptides M9-2, M10-3, or M10-4. Both 10 amino acid peptides, M10-3 and M10-4 contain the M9-2 sequence, with M10-3 having an additional glutamic acid at its N-terminus and M10-4 having an extra isoleucine at its C-terminal end. These peptides are located in a hydrophobic putative transmembrane domain in MART-1. The same lysis was observed when other HLA-A2$^+$ cells incubated with these peptides were used as targets including the K4B (provided by Dr. William Biddson, NIH; Storkus. W et al. (1993) J. of Immunology 151:3719-3727) and 501EBVB Epstein-Barr virus transformed B cells (Topalian et al. (1989) J. Immunol. 142: 3714-3725) or HMY-C1R B cells (Dr. William Biddson; NIH; Storkus, W. et al., (1993) J. of Immunol. 151:3719-3727) transfected with the HLA-A2.1 gene (data not shown).

The peptides, M9-1, M9-2, M9-3, M10-2, M10-3, M10-4 and M10-5 were further purified and titrated in order to evaluate their relative ability to sensitize T2 cells to lysis by MART-1 reactive TIL1235 or T cell clone A42 (Figure 2). The purified peptides M9-2, M10-3 and M10-4 were required in minimum concentrations of 1ng/ml, 100ng/ml an 1000ng/ml, respectively. The purified M10-4 was not recognized by TIL clone A42 even at 10ug/ml as shown in Figure 2. M9-1, M9-3, M10-2, and M10-5 peptides were not recognized by either A42 or TIL1235.

Table 5. Lysis of T2 cells preincubated with synthetic MART-1 peptides

Target	Peptide		A42	TIL 1235	TIL 660	TIL 1074
			% specific lysis			
501mel	none		47	30	31	41
397mel	none		1	0	1	2
T2	none		-2	-3	-1	1
T2	M9-1	TTAEEAAGI	-10	-5	-5	-4
T2	M9-2	AAGIGILTV	64	80	40	56
T2	M9-3	GIGILTVIL	18	20	0	10
T2	M9-4	GILTVILGV	1	-1	-3	2
T2	M9-5	ILTVILGVL	-2	-1	-5	-1
T2	M9-6	LTVILGVLL	1	0	1	0
T2	M9-7	TVILGVLLL	-2	-3	-2	1
T2	M9-8	VILGVLLLI	1	5	-2	-2
T2	M9-9	ALMDKSLHV	-1	-4	-8	0
T2	M9-10	SLHVGTQCA	-1	1	-8	4
T2	M9-11	PVVPNAPPA	-2	0	4	-1
T2	M9-12	NAPPAYEKL	1	-2	0	6
T2	M10-1	YTTAEEAAGI	-4	-2	-3	3
T2	M10-2	TAEEAAGIGI	7	11	12	15
T2	M10-3	EAAGIGILTV	55	66	31	51
T2	M10-4	AAGIGILTVI	34	68	29	21
T2	M10-5	GILTVILGVL	-1	2	7	10
T2	M10-6	ILTVILGVLL	1	6	6	7
T2	M10-7	LTVILGVLLL	-2	-1	-1	2
T2	M10-8	TVILGVLLLI	-6	-1	-1	11
T2	M10-9	RALMDKSLHV	3	5	8	11
T2	M10-10	SLHVGTQCAL	-2	-8	2	9
T2	M10-11	SLQEKNCEPV	3	2	2	9

Twenty-three peptides (SEQ ID NOS:3-25) (12 9-mers and 11 10-mers)(>90% purity) were synthesized and the lysability by TIL clone A42, TIL lines TIL1235, TIL660, and TIL1074 derived from different patients was tested against HLA-A2$^+$ T2 cells preincubated with each peptide (10ug/ml) in a 4h-^{51}Cr release cytotoxicity assay at E:T ratio of 20:1 for A42 and 40:1 for other TIL lines. T2 cells were lysed well when incubated with M9-2, M10-3 and M10-4. M10-3 and M10-4 contain the entire M9-2 sequence (underlined).

Recognition of MART-1 peptides by HLA-A2 restricted TIL established from different patients.

To evaluate whether a variety of HLA-A2 restricted MART-1 specific TIL recognized the same or different epitopes in the MART-1 antigen, lysis of T2 cells (Cerundolo V., et al. (1990) Nature 345: 449-452) preincubated with each peptide was tested with TIL derived from 10 melanoma patients. A representative experiment with 10 TIL is shown in Table 6. M9-2 and M10-3 were recognized by 9 of 10 TIL (only TIL1200 were negative) as well as the A42 clone with the same pattern of lysis as COS7 cells cotransfected with cDNAs encoding MART-1 and HLA-A2.1. Only TIL620 and TIL1088 demonstrated low level of non-specific lysis of T2 cells without peptides or after the addition of irrelevant peptides, but showed significant increase of lysis of T2 cells preincubated with M9-2, M10-3, and M10-5 peptides. The recognition of M10-4 differed among the TIL, but was similar to the different reactivity to M10-4 by the T-cell clone A42 or the T-cell line TIL1235 (Figures 2A and 2B). Higher concentrations (1ug/ml) of M10-4 were required for lysis than were required for M9-2 or M10-3. These 10 TIL and clone A42 also secreted cytokines including IFN-γ, GM-CSF and TNF-α when incubated with T2 cells preincubated with M9-2 or M10-3 (data not shown). Therefore, M9-2 or M10-3 are common epitopes recognized by a majority of HLA-A2 restricted melanoma specific TIL.

Table 6. Recognition of MART-1 peptides by HLA-A2 restricted melanoma specific TIL

Target	Peptide (ug/ml)	TIL 501	TIL 620	TIL 660	TIL 1074	TIL 1088	TIL 1128	TIL 1143	TIL 1200	TIL 1235	TIL 1363	clone A42
		% specific lysis										
501mel	none	42	49	35	32	31	19	24	41	32	43	41
397mel	none	3	16	6	1	1	4	3	3	3	4	1
T2	none	0	7	-3	-6	7	-6	-7	-6	-7	-7	-6
T2	M9-1 (1)	4	15	-4	1	31	1	-5	-1	1	4	3
T2	M9-2 (1)	86	75	73	79	98	30	36	2	92	82	91
T2	M9-2 (0.001)	52	49	23	32	81	9	6	1	10	41	63
T2	M9-3 (1)	5	25	0	1	19	0	1	-2	0	-2	-4
T2	M10-2 (1)	10	22	5	8	21	8	3	7	7	7	6
T2	M10-3 (1)	84	68	68	73	79	24	27	1	42	67	62
T2	M10-3 (0.001)	91	50	33	25	86	13	14	0	14	39	1
T2	M10-4 (1)	83	47	16	35	80	6	3	1	14	53	0
T2	M10-4 (0.001)	0	11	3	0	14	4	-1	-1	2	-3	-3
T2	M10-5 (1)	4	14	1	4	13	2	3	0	3	0	2

Lysability by TIL clone A42 and TIL lines derived from 10 patients of T2 cells preincubated with the purified peptides M9-1, M9-2, M9-3, M10-2, M10-3, M10-4, and M10-5 was tested in a 4h-^{51}Cr release assay at an E:T ratio of 20:1 for A42 and 40:1 for other TIL lines. Nine of ten TIL lysed T2 cells incubated with peptides M9-2 or M10-3. Seven of ten TIL lysed T2 incubated with peptide M10-4 at a concentration of 1ug/ml.

The relative frequency of recognition of known melanoma proteins by T-cells derived from the TIL of ten melanoma patients has been examined. The common epitopes, M9-2 and M10-3 in the MART-1 antigen that were dominantly recognized by nine of these TIL have also been identified. The cDNA encoding MART-1 was isolated by cDNA expression cloning using TIL1235 in screening assays (See Example 1). MART-1 is a 118 amino acid protein containing a single transmembrane domain and is expressed in most melanoma cells as well as cultured melanocytes and retina similar to the expression pattern of the cDNA for a form of gp100 described in Example 3. The gp100 is recognized by 4 of 10 TIL.

Based on dose response analysis, peptide M9-2 most effectively sensitized T2 cells for lysis (Figure 2) suggesting that this peptide may be naturally processed and presented on tumor cells. The T-cells recognizing M9-2 may react with peptide M10-3 or M10-4 because the latter 10-mer peptides contain the 9 amino acid sequence of peptide M9-2. There is some difference in recognition of these 3 peptides by different TIL. For example, M10-4 was poorly recognized by the T-cell clone A42, but was well recognized by some TIL lines, although a higher concentration of M10-4 was necessary to observe the lysis. This may be due to the variation of TCR affinity for the M9-2 and M10-4 peptides in the context of HLA-A2, or alternatively, TIL lines may contain different T-cell clones which only recognize either M9-2 or M10-4. Peptides M10-3 and M10-4 may also be naturally processed and presented by tumor cells. The existence of multiple melanoma antigens presented by HLA-A2 has previously been suggested by analyzing the recognition of melanoma cell clones by a variety of T-cell clones (Knuth, A. et al. (1989), Proc. Natl. Acad. Sci. (USA) 86:2804, Wolfel, T. et al., 1989 J. Exp. Med. 170:797) or by analyzing HPLC

peptide fractions that were isolated from HLA-A2 melanoma cells (Slingluff, C. L. Jr. et al., (1993) J. Immunol. 150:2955, Storkus; W. J. et al., (1993) J. Immunol. 151:3719).

The observation that most HLA-A2 restricted TIL from melanoma patients recognize common MART-1 peptides but not gp75 suggests that the M9-2 or M10-3 MART-1 peptides may be more immunogenic in inducing T-cell responses in vivo than other known melanoma antigens. Some of the TIL used in this study were injected along with IL2 into autologous patients, and interestingly, all 4 TIL (620, 660, 1074, 1200) that recognize a gp100 protein (Figure 5A; SEQ ID NO: 27) effectively induced tumor regression (more than 50% reduction of tumor). All but TIL1200 also recognized MART-1.

Example 3

Identification of a Second Human Melanoma Antigen Recognized by Tumor Infiltrating Lymphocytes Associated with in Vivo Tumor Rejection

cDNA expression cloning

The cDNA25 clone encoding a form of the melanoma antigen designated gp100 was cloned by techniques similar to those described in Example 1 and in Miki, T., et al. (1991) Proc. Natl. Acad. Sci. (USA) 88:5167-5171. Briefly, a breast cancer cell line, MDA231 (ATCC #HTB26), transfected with a cDNA library in λpCEV27 made from the 501mel melanoma cell line was screened for antigen positivity by measuring interferon-λ(IFN-γ) secretion when cocultured with TIL1200. TIL1200 was generated as described in Kawakami, Y., (1988), J. Exp. Med. 168, 2183-2191. The integrated cDNA was recovered from the genomic DNA of positive transfectants by PCR and cloned into the mammalian expression plasmid pCDNA3 (Invitrogen, San Diego, CA). The full length cDNA for cDNA25 was isolated

from the 501mel λpCEV27 library using the cDNA25 probe. The λphage containing the full length cDNA25 was digested with XhoI, and then self-ligated with T4 DNA ligase to make the plasmid pCEV27-FL25. Alternatively, a full length cDNA25 isolated by PCR using the specific primers designed for gp100 was cloned in pCRII(Invitrogen), and then cloned into pcDNA3(pcDNA3-FL25). To test whether this cDNA encoded a melanoma antigen it was retransfected into COS7, A375 or MDA231 and the resulting transfectants were tested for stimulation of TIL1200. DNA sequence of the plasmid clone pCEV27-FL-25 was determined with an automated DNA sequencer (Model 373A; Applied Biosystems, Inc.), using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc.) using the manufacturer's instructions.

Peptide synthesis and identification of antigenic peptides

Peptides were synthesized by a solid phase method using a Gilson AMS 422 multiple peptide synthesizer. The peptides were purified by HPLC on a Vydac C-4 column with 0.05% TFA/water-acetonitrile. To identify antigenic peptides, TIL lysis of T2 RET-cells preincubated with peptides for 2 hour (h) was measured using a ^{51}Cr release cytotoxicity assay.

Treatment of a patient with metastatic melanoma using TIL 1200

A 29 year old male patient, designated patient number 1200, with a widely metastatic melanoma who had previously failed chemotherapy and radiation therapy was treated with a single preparatory dose of 25 mg/Kg cyclophosphamide followed by the intravenous infusion of 1.6×10^{11} TIL (including 9.1×10^{9} Indium-111 labeled TIL) plus 7 doses of IL-2 at 720,000 IU/Kg given every 8 hours. A second cycle of treatment with TIL and IL-2 was given three weeks

later. Radionuclide scans showed localization of TIL in tumor deposits (Figure 3A). Biopsy of subcutaneous tumors on days 8 and 11 after treatment showed significant localization of TIL to tumor (ratios of injectate per gram in tumor compared to normal tissue were 14.9 and 14.0 respectively). The patient's cancer regressed rapidly following the first course of treatment. By three months after treatment 2 of 3 liver lesions had disappeared and a third lesion shrank by 50%. Multiple subcutaneous metastases regressed completely as shown in Figure 3B (the product of perpendicular diameters of individual lesions are shown).

Characterization of in vitro function of TIL1200

A number of TIL lines established from HLA-A2$^+$ melanoma patients lysed melanoma cell lines in a class I MHC-restricted fashion (Kawakami, Y., et al. (1992) J. Immunol. 148; 638-643), and were shown to release IFNγ, tumor necrosis factor-alpha (TNFα) or granulocyte-macrophage colony stimulating factor (GM-CSF) when cocultured with the same tumor cell lines (Hom, S.S., et al. (1993) J. Immunother. 13; 18-30). A CD8$^+$ CTL line, TIL1200, established from a metastatic subcutaneous tumor mass of patient 1200, lysed fresh autologous melanoma cells as well as 10 of 15 HLA-A2$^+$ allogeneic melanoma cell lines, but did not lyse 16 of 18 HLA-A2$^-$ melanoma cell lines or 6 of 8 HLA-A2$^+$ non-melanoma cell lines (Shilyansky, J., et al. (1993) Proc. Natl. Acad. Sci. USA, 91, 2829-2833, unpublished data). Table 7 shows a cytotoxicity assay against 5 representative HLA-A2$^+$ melanoma cell lines that were lysed by TIL1200, 4 representative HLA-A2$^+$ melanoma cell lines that were not lysed by TIL1200, and one HLA-A2$^-$ melanoma cell line. TIL1200 also secreted IFN-γ when cocultured with HLA-A2$^+$ normal cultured melanocytes established from neonatal

foreskin as well as HLA-A2$^+$ melanoma cell lines (Table 8). Therefore, TIL1200 appeared to recognize a non-mutated self peptide expressed in most melanomas and cultured neonatal melanocytes in an HLA-A2 restricted fashion.

Cloning of the cDNA coding for a melanoma antigen recognized by T cells

A cDNA library in λ pCEV27 from the HLA-A2$^+$ 501mel melanoma cell line, which was lysed by most HLA-A2 restricted melanoma specific TIL, was stably transfected into the highly transfectable HLA-A2$^+$ melanoma antigen negative MDA231 clone 7 or A375 clone 1-4. G418 resistant cells were selected and approximately 6700 individual transfectants from each cell line were isolated and screened based on their ability to stimulate IFN-γ secretion from TIL1200. Six DNA fragments were isolated by PCR using SP6/T7 primers flanking the integrated DNA from four MDA231 and one A375 transfectants that were positive in a second screening and were cloned into the mammalian expression vector pcDNA3 (Invitrogen).

These fragments in the pcDNA3 vector were transiently expressed in the COS7 cells with or without pcDNA3-HLA-A2.1. Transfection into COS7 of one of the cDNAs tested, cDNA25, along with HLA-A2.1 reproducibly conferred the ability to stimulate secretion of IFN-γ from TIL1200. The stable transfection of cDNA25 into A375 also stimulated IFN-γ release from TIL1200 (Table 9, Exp.1 and Exp. 2). A 2.2 Kb band detected by Northern blot analysis of the melanoma using the cDNA25 probe suggested that the cloned 1.6Kb fragment was not a full length cDNA. Comparison with the GenBank database of the consensus DNA sequence of

Table 7. Specificity of Antigen Recognition by TIL1200: Lysis of HLA-A2$^+$, gp100$^+$ Melanoma Cell Lines

Target	HLA-A2	gp100 FACS	gp100 Northern	TIL1200	LAK
				(% specific lysis)	
501mel	+	+	+	46	78
526mel	+	+	+	39	74
624mel	+	+	+	33	76
952mel	+	+	+	25	76
Malme3M	+	+	+	43	70
C32	+	-	-/+*	6	82
RPMI7951	+	-	-	9	67
WM115	+	-	-	5	68
HS695T	+	-	-	3	87
397mel	-	+	+	0	70

5 hour (h) ^{51}Cr release assay was performed to measure cellular cytotoxicity at an effector: target ratio of 40:1 as previously described(Kawakami, Y. et al. (1988) J. Exp. Med. 168:2183-2191). The expression of HLA-A2 and gp100 recognized by monoclonal antibody HMB45 (Enzo Diagnostics, New York, NY) was measured by flow cytometry(FACS). The expression of gp100 RNA was analyzed by Northern blot with a cDNA25 probe.

*-/+ indicates very weak positive.

Table 8. Specificity of Antigen Recognition by TIL1200: Recognition of HLA-a-a2$^+$ Neonatal Melanocytes

Stimulator	HLA-A2	TIL1200	TIL888
		(pgIFN-γ/ml)	
501mel	+	562	0
624mel	+	439	0
397mel	-	0	0
888mel	-	0	1970
NHEM493	-	441	0
NHEM527	+	418	0
NHEM530	+	164	0
NHEM616	+	53	0
FM725	+	107	0
FM801	+	250	343
NHEM483	-	0	0
NHEM680	-	0	0
HA002	-	0	0

The IFN-γ secretion by TIL was measured by ELISA as previously described in Example 1. The amount of IFN-γ secreted by TIL alone was subtracted (88 pg/ml for TIL888 and none for TIL 1200). TIL888 is a class I MHC restricted melanoma specific CTL, not restricted by HLA-A2. NHEM, FM, and HA refer to normal cultured melanocyte cell lines, all others are melanoma cell lines.

Table 9. Transfection of cDNA 25 into A375 and COS7

	Stimulator cells	Transfected genes	HLA-A2	Secretion by TIL1200
Exp. 1				(pgIFNγ/ml)
	501mel	none	+	987
	397mel	none	-	0
	A375	none	+	0
	A375	pcDNA3-25	+	230
Exp. 2				
	501mel	none	+	662
	397mel	none	-	0
	COS7	none	-	0
	COS7	HLA-A2.1	+	0
	COS7	pcDNA3-25	-	0
	COS7	HLA-A2.1+pcDNA3-25	+	310
Exp. 3				
	501mel	none	+	908
	397mel	none	-	0
	COS7	none	-	0
	COS7	HLA-A2.1	+	0
	COS7	pCEV27-FL25	-	0
	COS7	HLA-A2.1+pCEV27-FL25	+	742
	COS7	pcDNA3-FL25	-	0
	COS7	HLA-A2.1+pcDNA3-FL25	+	801

TIL 1200 secreted IFN-γ when coincubated with HLA-A2$^+$ A375 stably transfected with pcDNA3 containing truncated cDNA25 (pcDNA3-25) (Exp.1) or COS7 transiently transfected with either pcDNA3-25 (Exp. 2), pcDNA3 containing full length cDNA25 (pcDNA3-FL25) or pCEV27 containing full length cDNA25 (pCEV27-FL25) (Exp. 3) along with pcDNA3 containing HLA-A2.1 (HLA-A2.1). HLA-A2 expression was determined by flow cytometry and interferon-gamma secretion was measured by ELISA.

3 cDNA25 clones that were independently amplified by PCR revealed that cDNA25 was distinct from two previously registered genes, a gp100 (GenBank Access No. M77348) and Pmel17 (Kwon,B.S., et al. (1991) Proc. Natl. Acad. Sci, USA 88, 9228-9232). The cDNA 25 differed from the gp100 in GenBank (Accession No. M77348, also known as gp95) by two nucleotides, from the PMEL 17 sequence (Kwon et al. (1991) Proc. Natl. Acad. Sciences (USA) 58: 9228-9232) by 3 bases and a 21 base pair deletion. (Figure 5B).

The full length cDNA25(FL25) was isolated in two plasmids, pCEV27-FL25 or pCDNA3-FL25. Transfection of either plasmid into COS7 along with pcDNA3-HLA-A2.1 conferred to COS7 the ability to induce IFN-γ secretion by TIL1200. The amount of IFN-γ secretion stimulated by COS7 transfected with the full length DNA plus HLA-A2.1 was similar to that stimulated by 501mel and was higher than that stimulated by COS7 transfected with the truncated cDNA25 possibly due to improved translation starting at the normal AUG initiation codon (Table 9, Exp.2 and 3). Alternatively, the 5' region missing from the truncated cDNA25 may contain other epitopes recognized by clones in TIL1200. The requirement for HLA-A2.1 expression for IFN-γ release from TIL1200 and the fact that transfected cells did not stimulate IFN-γ secretion from irrelevant TIL (data not shown) demonstrated that the cDNA25 encoded an antigen recognized by TIL1200 in the context of HLA-A2.1 and did not encode a molecule that non-specifically induced IFN-γ release from T cells.

The nucleotide and corresponding amino acid sequences of the truncated cDNA 25 and the full length cDNA25 cloned from the 501mel cDNA library by screening with the cDNA25 probe (Figure 5A) were compared with the GenBank sequences of Pmel17 isolated from normal melanocytes and gp100 isolated from the melanoma cell line MEL-1. (Figure 5B). The full length cDNA25 differed from

the gp100 amino acid sequence at position 162. This amino acid difference is possibly caused by polymorphism or mutation in the tumor. cDNA25 had 2 amino acid differences at positions 162 and 274, compared to Pmel17 and did not contain 7 amino acids that existed in Pmel17 at positions 588-594. The amino acid sequence of the truncated cDNA25 that was isolated from the original MDA231 transfectant has a different sequence at the 3' end (from position 649 to the end) due to a frame shift caused by one extra cytidylic acid. It is not clear whether this difference was due to a true allelic difference or to a mutation that occurred during manipulation of the DNA. Nevertheless, TIL1200 appeared to recognize non-mutated peptides located between position 236 and 648. cDNA25 also had 87% similarity in amino acid sequence to cDNA RPE1 (Kim, R., and Wistow, G.J. (1992) Exp. Eye Res. 55: 657-662) specifically expressed in bovine retinal pigment epithelium and 60% similarity to cDNA MMP115 that encoded a melanosomal matrix protein isolated from chicken pigmented epithelial cells (Shilyansky, J., et al. (1993) Proc. Natl. Acad. Sci. USA, 91, 2829-2833).

A gp100 protein was known to be recognized by monoclonal antibody HMB45 (Adema et al., (1993) Am. J. Pathology, 143: 1579-1585). COS7 cells transfected with the full length cDNA25 were evaluated by flow cytometry using this monoclonal antibody. After transient expression of either pCEV27-FL25 or pcDNA3-FL25, COS7 expressed the antigen detected by HMB45 (data not shown).

Expression of RNA for cDNA25

Northern blot analysis was performed with the cDNA25 probe to evaluate the tissue specific expression of this gene. Ten of 15 melanoma cell lines and 6 of 6 melanocyte cell lines were positive for cDNA 25 (Figures 6A and 6B). Of many normal tissues tested only retina was positive (Figure 6C). Seven cell lines from T-cell (TILA, B), B-cells (501EBVB,

836EBVB) and fibroblast (M1) and 20 non-melanoma tumor cell lines (colon cancer, Collo, SW480, WiDr; breast cancer, MDA231, MCF7, HS578, ZR75; neuroblastoma, SK-N-AS, SK-N-SH; Ewing sarcoma, TC75, RD-ES, 6647; sarcoma 143B; glioma, U138MG, HS683; renal cell cancer, UOK108, UOK117, small cell lung cancer, H1092; Burkitt's lymphoma, Daudi; myeloma HMY) were all negative for cDNA25 (data not shown). Therefore, this gene appeared to be specifically expressed in melanocyte lineage cells, consistent with the expression pattern of previously isolated forms of gp100 when analyzed using monoclonal antibodies, HMB45, NKI/betab, or HMB-50 (Adema, G.J., et al. (1993) Am J Pathology 143: 1579-1585; Gown, A.M., et al., (1986) Am J Pathol 123:195-203; Colombari, R., et al. (1988) Virchows Archiv A Pathol Anat. 413:17-24); Vennegoor, C., et al. (1988) Am. J. Pathol. 130:179-192; Vogel, A.M., and Esclamado R.M. (1988) Cancer Res. 48:1286-1294). The levels of expression of the RNA detected by the cDNA25 probe in cultured neonatal melanocyte cell lines was significantly lower than that in melanoma cell lines. There was a perfect correlation between gp100 expression detected by Northern blot analysis with cDNA25 and flow cytometry using HMB45 antibody and melanoma lysis by TIL1200 in the 10 HLA-A2$^+$ melanoma cell lines as shown in Table 7.

Identification of the epitope in gp100

Based on a comparison of the amino acid sequence of the truncated form of cDNA 25 to known binding motifs of HLA-A2.1 (Falk, K., et al. (1992) Nature 351:290-296; Hunt, D.F., et al. (1992) Science 255:1261-1263; Ruppert, J., et al. (1993) Cell 74:929-9937,) 30 peptides of 9 or 10 amino acids in length from cDNA25 were synthesized. TIL1200 lysed the HLA-A2$^+$ cell line, T2, only when incubated with the peptide LLDGTATLRL (SEQ ID NO: 27 residues 457-486. Figure 5A; SEQ ID NO: 33) but not when

incubated with the other 29 peptides (Table 10, Figure 5A). Only peptide LLDGTATLRL (SEQ ID NO: 33) was able to also stimulate IFN-γ secretion by TIL 1200 (data not shown).

Many melanoma-specific CTL derived from TIL appear to recognize non-mutated self peptides derived from melanocyte-melanoma lineage specific proteins, since these TIL recognize most melanoma cell lines and normal cultured melanocytes sharing the appropriate restriction element. (Anichini, A., et al. (1993) J. Exp. Med. 177:989-998; Kawakami, Y., et al. (1993) J. Immunother. 14:88-93). In an attempt to isolate and identify melanoma antigens of value in the immunotherapy of melanoma patients, TIL, TIL1200 were used that, when transferred into a patient with metastatic cancer, localized to the tumor site and was associated with a dramatic tumor regression. It has been shown that, in contrast to non-activated lymphocytes and lymphokine activated killer cells, autologous TIL localize to tumor sites. This localization correlated with the ability of these TIL to mediate tumor regression (data not shown). TIL1200 which was a TIL line containing multiple CTL species recognized a tumor antigen in the context of HLA-A2, which is the most frequently expressed class I MHC antigen (about 50% of individuals) and has been shown to be a dominant restriction element for the induction of melanoma specific CTL. (Crowley, N.J., et al. (1991) J. Immunol. 146, 1692-1699).

By cDNA expression cloning using T cell recognition for screening, a cDNA (Figures 4A and 4B; SEQ ID NO: 26) encoding an antigen recognized by TIL1200 and identified as a form of gp100, a membrane glycoprotein also recognized by monoclonal antibodies, HMB45, HMB50 or NKI/betab has been identified. (Adema, G.J., et al. (1993) Am J Pathology 143, 1579-1585. Gown, A.M., et al. (1986) Am J Pathol 123, 195-203. Colombari, R., et al.

(1988) Virchows Archiv A Pathol Anat. 413, 17-24; Vennegoor, C., et al. (1988) Am. J. Pathol. 130, 179-192; Vogel, A.M., and Esclamado R.M. (1988) Cancer Res. 48, 1286-1294). These antibodies are highly specific for melanocyte lineage tissues and strongly stain most melanoma cells. NKI/betab also reacts with adult melanocytes in normal skin (Vennegoor, C., et al. (1988)

Table 10. TIL1200 Lysis of the T2 HLA-A2$^+$ Cell Line Pulsed with the Peptide, LLDGTATLRL

Target	HLA-A2	Peptide* (ug/ml)	TIL1200 (% specific lysis)**	TIL1235+
501mel	+	0	66	51
397mel	-	0	1	0
T2	+	0	2	1
T2	+	40	28	ND++
T2	+	10	32	0
T2	+	1	24	ND
T2	+	0.1	6	ND
T2	+	0.01	0	ND
T2	+	0.001	2	ND

* TIL1200 lysed T2 cells pulsed with the 10-mer peptide, LLDGTATLRL(457-466), but not other 29 peptides SEQ ID NO: 27 (residues 273-281, 297-306, 373-381, 399-407, 399-408, 409-418, 456-464, 463-471, 465-473, 476-485, 511-520, 519-528, 544-552, 544-553, 570-579, 576-584, 576-585, 585-593, 592-600, 597-605, 597-606, 602-610, 602-611, 603-611, 605-614, 606-614, 606-615, 619-627, 629-638)

\+ TIL1235 is an HLA-A2 restricted melanoma specific CTL that does not recognize gp100.

** E:T of 50:1

\++ ND, not done.

Am. J. Pathol. 130, 179-192). Immunoelectron-microscopic studies using either HMB45 or NKI/betab antibody revealed that a gp100 protein was mainly located in a membrane and filamentous matrix of stage I and II melanosomes in the cytoplasm (Vennegoor, C., et al. (1988) Am. J. Pathol. 130, 179-192; Schaumburg-Lever, G., et al. (1991) J. Cutan. Pathol. 18, 432-435). By a completely independent procedure, a cDNA encoding another form of gp100 was also isolated by screening with a rabbit polyclonal antiserum against gp100 (Adema, G.J., et al. (1993) Am J Pathology 143:1579-1585) and TIL1200 also lysed HLA-A2$^+$ cell lines transfected with this cDNA clone (Bakker, A.B.H. et al. (1994) J. Exp. Med. 179:1005-1009).

The existence of T cells reactive to the self-antigen gp100 in tumors and the possible enrichment of these T cells at the tumor site as a possible consequence of the specific accumulation and expansion of antigen reactive cells (Sensi, M. et al., (1993) J. Exp. Med 178:1231-1246) raises important questions about the nature of the immune response to self antigens on growing cancers and about the mechanisms of immunologic tolerance to self-antigens. The increased expression of gp100 on melanoma cells relative to that in melanocytes demonstrated by Northern blot analysis or the unique inflammatory conditions that might exist at the tumor site, which may be associated with the secretion of cytokines and expression of costimulatory molecules on the cell surface, could break tolerance to gp100. Depigmentation has been reported to be associated with a good prognosis (Nordlund, J.J., et al. (1983) J. Am. Acad. Dermatol. 9:689-695; Bystryn, J-C, et al. (1987) Arch. Dermatol., 123:1053-1055) and with clinical response to chemoimmunotherapy (Richards, J.M., et al. (1992) J. Clin. Oncol. 10:1338-1343) in melanoma patients. Sporadic vitiligo in has been seen patients receiving melanoma-specific TIL but adverse ophthalmologic effects

that might be related to melanocyte destruction has not been observed. Patient 1200 did not develop vitiligo or any ophthalmologic side-effects.

The gp100 protein (Figure 5A; SEQ ID NO: 27) and the ten amino acid peptide identified may represent a human tumor rejection antigen since the transfer into patient 1200 of TIL1200 plus IL2 was associated with cancer regression. The traffic of TIL1200 to tumor deposits in vivo and the rapidity of the antitumor response are characteristics of the response to TIL therapy, although IL2 may also have been involved in the tumor rejection. Adoptive transfer of 3 other TIL lines which recognized gp100 as well as MART-1 also mediated tumor regression (data not shown).

Tyrosinase (Brichard, V., et al. (1993) J. Exp. Med. 178, 489-495) and MART-1 (see Example 1) have been identified as melanoma antigens recognized by HLA-A2 restricted CTL. Another antigen, MAGE-1 is recognized by HLA-A1 restricted melanoma-specific CTL and is expressed on a variety of cancer cells as well as testis (Van Der Bruggen, P. et al. (1991) Science, 254:1643-1647). However, none of the ten HLA-A2 restricted TIL recently developed appeared to recognize MAGE-1 (Zakut, R., et al. (1993) Cancer Res. 53: 5-8).

The wide expression of gp100 proteins in melanomas, the recognition of a peptide by T cells infiltrating into tumor, its restriction by HLA-A2, present in 50% of individuals, and the association of anti gp100 reactivity with cancer regression in patient 1200 imply that the gp100 antigen in particular the novel immunogenic peptides derived from the gp100 amino acid sequence (Figure 5A; SEQ ID NO: 27) may be particularly useful for the development of active immunotherapies for patients with melanoma.

Example 4

Recognition Of Multiple Epitopes in Human Melanoma Antigen by TIL Associated with In Vivo Tumor Recognition

Materials and Methods

Generation of CTL from TIL and Treatment of Patients with Metastatic Melanoma

Melanoma specific CTL were induced and expanded from TIL in media containing 6000IU/ml of IL2 as previously described (Kawakami, et al., (1988) *J. Exp. Med.* 168:2183). All available HLA-A2 restricted melanoma specific CTL which were administered to autologous patients in the Surgery Branch, NCI, were used in this study. TIL were administered intravenously along with IL2 into autologous patients with metastatic melanoma as previously reported (Rosenberg, S. A., et al., (1988) *N Engl J Med* 319:1676; Rosenberg S.A., et al., (1994) J. NCI. 86:1159). Fisher's exact test was used to determine the association of gp100 recognition by TIL with clinical response to TIL treatment; likewise with MART-1 recognition.

Synthesis of Peptides

Peptides were synthesized by a solid phase method using a peptide synthesizer (model AMS 422;Gilson Co.Inc., Worthington, OH)(>90% purity). The peptides to be synthesized were selected from the reported human sequence of gp100 based on HLA-A2.1 binding motifs (Falk, K., (1991) *Nature* 351:290; Hunt, D. F., et al, (1992) *Science* 255:1261; Ruppert, J., et al., (1993) *Cell* 74:929; Kubo, RT, et al. (1994) *J Immunol.* 152:3913). The following peptides were tested: Eight 8-mer peptides (with residues starting at -199, 212, 218, 237, 266, 267, 268, 269; see Figure 7A), eighty-four 9-mer peptides with residues starting at - 2, 4, 11, 18, 154, 162, 169, 171, 178, 199, 205, 209, 216, 241, 248, 250, 255, 262, 266, 267, 268, 273, 278, 280, 273, 286, 287, 298, 290, 309, 316, 332,

335, 350, 354, 358, 361, 371, 373, 384, 389, 397, 399, 400, 402, 407, 408, 420, 423, 425, 446, 449, 450, 456, 463, 465, 485, 488, 501, 512, 536, 544, 563, 570, 571, 576, 577, 578, 583, 585, 590, 592, 595, 598, 599, 601, 602, 603, 604, 606, 607, 613, 619, 648; see Figure 7A) and seventy-seven, 10-mer peptides with residues starting at -9, 17, 57, 87, 96, 154, 161, 169, 177, 197, 199, 200, 208, 216, 224, 232, 240, 243, 250, 266, 267, 268, 272, 285, 287, 289, 297, 318, 323, 331, 342, 350, 355, 357, 365, 380, 383, 388, 391, 395, 399, 400, 406, 407, 409, 415, 432, 449, 453, 457, 462, 476, 484, 489, 492, 511, 519, 536, 543, 544, 548, 568, 570, 571, 576, 577, 584, 590, 595, 598, 599, 601, 602, 603, 605, 611, 629; see Figure 7A) were synthesized. Possible epitopes identified in the first screening were further purified by HPLC on a C-4 column (VYDAC, Hesperia, CA)(>98% purity) and the molecular weights of the peptides were verified by mass spectrometry measurement as previously described (Example 3; Kawakami, Y., et al., (1994) *J.Exp.Med.* 180:347; Kawakami, Y., et al., (1994) *Proc Natl Acad Sci (USA)* 91:6458).

Peptide Binding Assay to HLA-A2.1

Soluble HLA-A2.1 heavy chain, human beta 2-microglobulin, radiolabeled peptide HBc_{18-27} (FLPSDYFPSV) and various concentrations of the sample peptides were coincubated in the presence of protease inhibitors for 2 days at room temperature as previously described (Ruppert, J., et al., (1993) *Cell* 74:929; Kubo, RT, et al., (1994). *J Immunol.* 152:3913; Sett A., et al., (1994). Molecular Immunol. 31:813). The percentage of labeled peptide bound to HLA-A2.1 was calculated after separation by gel filtration and the concentration of the sample peptide necessary to inhibit 50% of the binding of the labeled peptide was calculated. The relative affinity of peptides to HLA-A2.1 were also calculated as a ratio (concentration

of the standard HBc_{18-27} peptide to inhibit 50% of the binding of the labeled peptide / concentration of the sample peptide to inhibit 50% of the binding of the labeled peptide) as previously described (Sett A., et al., (1994) Molecular Immunol. 31:813). Peptide binding was defined as high (50% inhibition at <50nM, ratio >0.1), intermediate (50-500nM, ratio 0.1-0.01) or weak (>500nM, ratio <0.01) (Ruppert, J., et al., (1993) *Cell* 74:929; Kubo, RT, et al., (1994) *J Immunol.* 152:3913; Sett A., et al., (1994) Molecular Immunol. 31:813).

The pcDNA3 plasmid containing the full length gp100 cDNA (Example 3; Kawakami, Y., et al., (1994)). *Proc Natl Acad Sci (USA)* 91:6458) was digested with Xho I and Xba I. After incorporation of alpha-phosphorothioate deoxynucleoside triphosphate into the Xba I site, a standard exonuclease III nested deletion was performed using the Exo Size Deletion Kit (New England Biolabs, inc., Beverly, MA). The deleted clones were self-ligated and amplified. The exact deletion for each clone was confirmed by DNA sequencing. To identify the region containing epitopes, pcDNA3 plasmids (Invitrogen, San Diego CA) containing the cDNA fragments (D3, D5, D4, C3) generated by the sequential deletion with exonuclease from the 3' end of the full length gp100 cDNA as well as the truncated gp100 cDNA lacking the 5'-coding region (25TR) (Example 63; Kawakami, Y. (1994) *Proc Natl Acad Sci (USA)* 91:6458), were transfected into COS7 cells along with the HLA-A2.1 cDNA and the recognition of the transfected COS cells by TIL was evaluated using IFN-γ release assays (Example 1; Kawakami, Y., (1994) *Proc Natl Acad Sci (USA)* 91:3515).

Evaluation of Antigen Recognition by T-cells

To assess antigen recognition by T-cells, a ^{51}Cr release assay or an IFN-γ release assay were performed as previously described (Examples 1 and 2; Kawakami, Y., et

al., (1994)). Proc Natl Acad Sci (USA) 91:3515; Kawakami, Y., et al., (1988). J. Exp. Med. 168:2183). Either COS7 cells transfected with cDNA encoding melanoma antigens and HLA-A2.1 cDNA, or T2 cells preincubated with peptides were used as stimulators for the IFN-γ release assay. T2 cells pulsed with peptides were also used as targets for cytotoxicity assays (Kawakami, Y., (1994) J.Exp.Med. 180:347).

Recognition of gp100 by TIL correlated with clinical response to TIL treatment

Four of 14 HLA-A2 restricted melanoma specific CTL derived from TIL recognized gp100 while 13 recognized MART-1 (3 recognized both gp100 and MART-1). None recognized tyrosinase or gp75 as assessed by the reactivity of TIL against COS7 cells transfected with the cDNA encoding these melanoma antigens along with HLA-A2.1 cDNA (Example 2; Kawakami, Y et al. (1994) J. Exp. Med. 180:347). The HLA-A2 restriction and the recognition specificity of these 4 gp100 reactive CTL has been previously demonstrated (Examples 1-3; Kawakami, Y., et al., (1994) Proc Natl Acad Sci (USA) 91:6458; Kawakami, Y., et al., (1992) J Immunol 148:638; O'Neil, B. H., et al., (1993) J Immunol 1410:1418; Shilyansky, J., et al., (1994) Proc. Natl. Acad. Sci. (USA) 91:2829). Ten of these 14 CTL were administered into the autologous patients along with IL2. As summarized in Table 11, all 4 patients treated with CTL capable of recognizing gp100 resulted in an objective partial response (>50% tumor regression). Clinical response to TIL therapy associated with reactivity of TIL to gp100 (p=0.0048) but not to MART-1 (p=0.4). These data suggested that gp100 may contain epitopes capable of mediating in vivo tumor regression.

Identification of epitopes recognized by gp100 reactive TIL

To identify the epitopes recognized by these 4 gp100 reactive CTL, a 169 peptides which contained HLA-A2.1 binding motifs were synthesized. Peptide recognition was evaluated by testing the reactivity of these CTL against HLA-A2.1+ T2 cells preincubated with each peptide using both cytotoxicity and IFN-γ release assays. As shown in Table 12, 7 peptides were recognized by gp100 reactive TIL in the cytotoxicity assays. The results of the IFN-γ release assays performed at the same time were consistent with that of the cytotoxicity assays. The different subcultures of TIL620 (620-1, 620-2) or TIL660 (660-1, 660-2, 660-3) were grown from the TIL culture that was administered into the autologous patient, but they were separately cultured and had slightly different specificities likely due to the in vitro expansion of different clones. $G9_{209}$ (ITDQVPFSV) (SEQ ID NO:48) and $G10_{208}$ (TITDQVPFSV) (SEQ ID NO:49), which has an extra threonine at the N-terminus of $G9_{209}$, were recognized only by TIL620. $G9_{154}$ (KTWGQYWQV) (SEQ ID NO:46) and $G10_{154}$ (KTWGQYWQVL) (SEQ ID NO:47), which has an extra leucine at the C-terminus of $G9_{154}$, were recognized by TIL1200, TIL620-2 and TIL660-2. G10-4 (LLDGTATLRL) (SEQ ID NO:33) was recognized by TIL1200 as demonstrated (Example 3). The peptide $G9_{280}$ (YLEPGPVTA) (SEQ ID NO:40) was recognized by TIL660 and TIL1143. TIL660-3 also recognized G10-5 (VLYRYGSFSV) (SEQ ID NO:34) as well as $G9_{280}$. Lysis of T2 cells preincubated with G10-5 was repeatedly low, possibly because a small subset of T-cell clones was specific for this epitope.

To complement the epitope identification using the known HLA-A2.1 binding motifs, another method was also used to identify regions possibly containing epitopes. Five gp100 cDNA fragments, 4 generated by exonuclease

deletion from the 3'-end of the cDNA (D3, D4, D5, C4) as well as a partial cDNA clone lacking the first 705 base pairs of the 5'-coding region (25TR), were inserted into the pcDNA3 plasmid and transfected into COS7 cells along with the HLA-A2.1 cDNA. The locations of the fragments are shown in Figure 7A. The recognition of these transfectants by the 4 gp100 reactive TIL was evaluated using an IFN-γ release assay (Figure 7B). TIL 1200 recognized COS cells transfected with the fragments, 25TR, D5, D4 or C4, but not with D3, suggesting that at least 2 epitopes existed in the regions of amino acid residues 146-163 and 236-661. $G9_{154}$ and $G10_{154}$ were the only peptides which contained HLA-A2.1 binding motifs in the region 146-163 and both were recognized by TIL1200. G10-4 was located in the region 236-661 and was recognized by TIL1200. TIL620-1 recognized COS cells transfected with C4 but not with D3, D5, D4 or 25TR, suggesting that the epitope existed within residues 187-270. $G9_{209}$ and $G10_{208}$ which were recognized by TIL620-1 were located in this region. TIL620-2, another subculture of TIL620, also recognized COS cells transfected with D5 and D4, but not D3, and recognized $G9_{154}$ and $G10_{154}$ in the region 147-163, also recognized by TIL1200. TIL660-1 and TIL1143 recognized COS cells transfected with C4 or 25TR, but not with D3, D5, or D4, suggesting that epitopes existed in the 2 regions 187-270 and 236-661. $G9_{280}$ located in the fragment 25TR, but not in the fragment C4, was recognized by TIL660 and TIL1143.

Binding Affinity of the Melanoma Epitopes to HLA-A2.1 in Vitro

With the exception of G10-4, which required a concentration of 1ug/ml to sensitize T2 cells for CTL lysis (Example 3; Kawakami, Y., et al., (1994) *Proc Natl Acad Sci (USA)* 91:6458), all gp100 epitopes identified in this study could sensitize T2 cells for CTL lysis at a

concentration of 1ng/ml (Figures 8A-8D). G10-5 appeared to be inhibitory to the cytotoxic activity of CTL at concentration greater than 10ng/ml since lysis of T2 cells incubated with G10-5 at more than 10ng/ml was repeatedly lower than at 1-10ng/ml in this assay condition in which the peptide was present in the medium during entire 4h cytotoxicity assay (Figure 8D). The relative binding affinity of these epitopes to HLA-A2.1 was also measured using an in vitro competitive binding assay (Table 13). $G9_{154}$ had an higher binding affinity (50% inhibition of the standard peptide at 11nM) to the HLA-A2.1 molecule than $G10_{154}$ (1010nM) which contains an extra leucine at the C-terminus of $G9_{154}$, and could sensitize T2 cells at lower concentrations than $G10_{154}$ (Figure 8A). $G9_{209}$ also bound to HLA-A2.1 with higher affinity (84nM) than $G10_{208}$ (2080nM) which contains an extra threonine at the N-terminus, and could sensitize T2 cells at lower concentrations of peptide than $G10_{208}$(Figure 8B). Thus, the 9-mer peptides were superior to the corresponding 10 mer peptides in the sensitization of T2 cells to CTL lysis, and they also had higher binding affinities to HLA-A2.1. This was also the case for the identified MART-1 9 and 10 amino acid peptides (M9-2, M10-3, M10-4) (Example 2; Kawakami, Y., et al., (1994). *J.Exp.Med.* 180:347). The results of the peptide titration in the T2 cell lysis assay correlated with the results of the HLA-A2.1 binding affinity as measured by the in vitro binding assay. The other gp100 epitopes, $G9_{280}$, G10-4, or G10-5 had binding affinities for HLA-A2.1 with 50% inhibition at 95nM, 483nM, or 13nM, respectively. The HLA-A2.1 binding affinities of the previously identified HLA-A2 restricted melanoma epitopes in MART-1 (Example 2; Kawakami, Y., et al., (1994) *J.Exp.Med.* 180:347) and tyrosinase (Wolfel, T., (1994) *Eur.J.Immunol.* 24:759; SEQ ID NOS: 31 and 32) were also measured (M9-2(397nM), M10-3(2272nM), M10-4(5555nM), $T9_1$

(333nM), $T9_{369}$ (40nM)). Except for the 10mer peptides ($G10_{154}$, $G10_{208}$, M10-3, M10-4), for which overlapping 9-mer epitopes ($G9_{154}$, $G9_{209}$, M9-2) existed, all melanoma epitopes had either high ($G9_{154}$, G10-5, $T9_{369}$) or intermediate ($G9_{209}$, $G9_{280}$, G10-4, M9-2, $T9_1$) binding affinities to HLA-A2.1.

Discussion

Multiple epitopes in the gp100 human melanoma antigen recognized by 4 TIL which were associated with tumor regression when adoptively transferred to the autologous patients have been identified in this study. Among the 5 epitopes described in this study, $G9_{154}$ or $G10_{154}$ appeared to be the most commonly recognized, since these were recognized by 3 of 4 gp100 reactive TIL derived from different patients. Although the $G9_{280}$ peptide was reported to be recognized by all 5 CTL derived from PBL of different patients (Cox, A.L., et al., (1994)). *Science* 264:716), it was only recognized by 2 of 4 gp100 reactive TIL in this study. This difference may be due to the sources of T-cells (TIL vs PBL) used.

It will be appreciated that the MART-1 peptide M9-2 may also be designated $M9_{27}$, the MART-1 peptide M10-3 may also be designated $M10_{26}$, and the MART-1 peptide M10-4 may also be designated $M10_{27}$. It will also be appreciated that the gp100 peptide G10-4 may also be designated $G10_{457}$ and the gp100 peptide G10-5 may also be designated $G10_{476}$.

Table 11. Summary of antigen recognition by HLA-A2 restricted melanoma specific TIL

	TIL									
	1200	620	660	1143	1074	1088	1235	1318	1363	1399
Clinical* Response	PR	PR	PR	PR	NR	NR	NR	NR	NR	NR
Antigen (epitope)										
gp100	+ ($G9_{154}$) ($G10\text{-}4$)	+ ($G9_{209}$) ($G9_{154}$)	+ ($G9_{280}$) ($G9_{154}$) ($G10\text{-}5$)	+ ($G9_{280}$)	-	-	-	-	-	-
MART-1	-	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)	+ (M9-2)
tyrosinase	-	-	-	-	-	-	-	-	-	-
gp75	-	-	-	-	-	-	-	-	-	-

G9(10): gp100-9(10)-mer peptides, M9-2: MART-$1_{27\text{-}35}$ peptide
Recognition of gp100 by TIL is significantly ($p<0.001$) correlated with clinical response for adoptive immunotherapy with HLA-A2 restricted TIL.

* PR, partial response (>50% reduction in the sum of the product of perpendicular tumor diameters); NR, no response (<50 % reduction)

Table 12. Recognition of gp100 peptides by TIL

Target cells	Peptide	TIL (% specific lysis at E:T=40:1)					
Exp. 1		620-1	620-2	660-1	1143	1200	1235
624mel	none	**32**	**36**	**47**	**20**	**77**	**11**
397mel	none	2	3	0	0	0	0
T2	none	0	5	3	1	0	2
T2	M9-2	**19**	**84**	**69**	**49**	1	**86**
T2	$G9_{154}$	0	**21**	4	0	**100**	0
T2	$G10_{154}$	3	**19**	7	4	**75**	2
T2	$G9_{209}$	**45**	**21**	0	3	0	0
T2	$G10_{208}$	**42**	**36**	7	4	2	3
T2	$G9_{280}$	2	7	**43**	**11**	0	0
T2	G10-4	0	7	6	0	**15**	0
T2	G10-5	2	7	2	1	7	0
Exp. 2		620-1	620-2	660-2	1143	1200	1235
624mel	none	**60**	**65**	**74**	**49**	**82**	**18**
397mel	none	2	6	0	0	0	0
T2	none	1	12	1	0	1	2
T2	M9-2	**36**	**85**	**50**	**39**	0	**60**
T2	$G9_{154}$	5	**27**	**32**	1	**78**	5
T2	$G10_{154}$	4	**31**	**30**	2	**85**	3
T2	$G9_{209}$	**22**	**74**	5	4	1	3
T2	$G10_{208}$	**35**	**80**	7	**10**	1	5
T2	$G9_{280}$*	2	9	**75**	**34**	1	2
Exp. 3				660-3	1143	1200	1235
624mel	none			**52**	**15**	**66**	**40**
397mel	none			5	3	7	4
T2	none			7	3	7	4
T2	M9-2			**50**	**62**	4	**94**
T2	$G9_{280}$			**99**	**37**	9	5
T2	G10-4			0	0	**50**	0
T2	G10-5			**14**	2	6	5

Lysis by TIL of T2 cells preincubated with MART1 epitope, M9-2 (AAGIGILTV) and gp100 epitopes, $G9_{154}$ (KTWGQYWQV), $G10_{154}$ (KTWGQYWQVL), $G9_{209}$(ITDQVPFSV), $G10_{208}$ (TITDQVPFSV), $G9_{280}$ (YLEPGPVTA), G10-4(LLDGTATLRL), G10-5 (VLYRYGSFSV) at 1ug/ml (* 1ng/ml), was measured by 4h-^{51}Cr release assays. TIL620-1, -2 or TIL660-1, -2, -3 were grown from the same TIL which was administered into the autologous patient, but were separately cultured. 624mel, HLA-A2+ gp100+, MART-1+ melanoma cell line, 397mel, HLA-A2- melanoma cell line. T2 cells, HLA-A2+ T cell-B-cell hybridoma. Bold: statistically significant lysis

Table 13. The relative binding affinity of the human melanoma epitopes to HLA-A2.1

Protein	Peptide	Sequence	50% inhibition (nM)[a]	Ratio to Standard.[b]
gp100	$G9_{154}$	KTWGQYWQV	11	0.45
	$G10_{154}$	KTWGQYWQVL	1010	0.005
	$G9_{209}$	ITDQVPFSV	84	0.06
	$G10_{208}$	TITDQVPFSV	2080	0.0024
	$G9_{280}$	YLEPGPVTA	95	0.053
	G10-4	LLDGTATLRL	483	0.01
	G10-5	VLYRYGSFSV	13	0.38
MART-1	M9-2	AAGIGILTV	395	0.013
	M10-3	EAAGIGILTV	2272	0.0022
	M10-4	AAGIGILTVI	5555	0.0009
Tyrosinase	$T9_{1}$	MLLAVLYCL	333	0.015
	$T9_{369}$	YMNGTMSQV	40	0.13

[a] Concentration of sample peptide required for 50% inhibition of the standard radiolabeled peptide HBC18-27.

[b] Ratio of the binding affinity of the sample peptide to that of the standard peptide (50% inhibition at 5nM). Peptides are defined as high (50% inhibition at <50nM, ratio >0.1), intermediate (50-500nM, ratio 0.1-0.01) and weak (>500nM, ratio <0.01) binding peptides.

Example 5

Modification of melanoma epitopes for improvement of immunogenicity

Material and Methods

Peptide synthesis and HLA-A2.1 binding assay. Peptides were synthesized by a solid phase method using a multiple peptide synthesizer and purified by HPLC, as previously described (Rivoltini, L et al. (1995) Journal of Immunology Volume 154:2257-2265). The relative binding of peptides to HLA-A2.1, based on the inhibition of binding of a radiolabeled standard peptide to detergent-solubilized MHC molecules, was performed as previously described (Rivoltini, L et al. (1995) Journal of Immunology Volume 154:2257-2265). Briefly, various doses of the test peptides (ranging from 100 μM to 1 nM) were coincubated together with the 5nM radiolabeled Hbc 18-27 (FLPSDYFPSV) (SEQ ID NO: 125) peptide and HLA-A2.1 heavy chain and β2-microglobulin for 2 days at room temperature in the presence of protease inhibitors. The percentage of MHC-bound radioactivity was determined by gel filtration and the 50% inhibitory dose was calculated for each peptide.

Induction of peptide specific CTL PBMC were separated from peripheral blood of HLA-A2+ melanoma patients and normal donors by centrifugation on Ficoll-Hypaque gradients and used as fresh or cryopreserved samples. Peptide specific CTL lines were generated as follows: at day 0, PBMC were plated at a concentration of 1.5×10^6/ml in 24-well plates (2ml/well) in Iscove's medium containing 10% human AB serum, L-glutamine, antibiotics (CM) and in the presence of 1 ug/ml peptide. Two days later, 12 IU/ml interleukin 2 (IL-2) (Chiron Co., Emeryville, CA) were added to the cultures. Lymphocytes were then restimulated weekly as follows: responder cells were harvested, washed once and replated in 24-well plates at a concentration of 2.5×10^5

cells/ml in CM. Autologous PBMC were thawed, washed twice in PBS, resuspended at 5-8x10^{6}cells/ml in CM and pulsed with 1ug/ml peptide in 15-ml conical tubes (5ml/tube) for 3 hours at 37°C. These PBMC (stimulators) were then irradiated at 3000 rads, washed once in PBS and added to the responder cells at responder:stimulator ratios ranging between 1:3 and 1:10. The next day, 12 IU/ml IL-2 were added to the cultures. The activity of these CTL was tested by cytotoxicity assays after at least 2 rounds (14 days) of peptide stimulation. To generate CTL from TIL cultures, the dissociated tumor suspension were cultured for 1-2 days in 10% FCS RPMI-1640 medium to allow tumor cell adherence. The lymphocytes, recovered from the non-adherent fraction, were used for the induction of peptide specific CTL as described above.

Assessment of antigen recognition by CTL. ^{51}Cr release cytotoxic assays were performed to detect the recognition of peptide and melanoma cells by CTL. To analyze peptide recognition, T2 cell lines were preincubated for 2 h at 37°C with 1ug/ml peptide, washed and used as target cells in a ^{51}Cr release cytotoxic assay. The melanoma lines 624mel was established in our laboratory (See Example 1).

In order to make more immunogenic peptides for induction of anti-melanoma T-cells than natural melanoma epitopes, a variety of peptides in which at least 1 amino acid was changed based on consensus motifs in peptides binding to a specific MHC Class I allele Falk, et al. (1991) Nature 351:290; Kubo et al. (1994) J. Immunol 152:3913; Parker, K. et al. (1992) Journal of Immunology 149:3580; Ruppert, J. et al. (1993) Cell 74:929-937) (Tables 14, 15, 16, and 17). Although most of the previously isolated viral epitopes and the naturally processed HLA-A2.1 binding peptides contained leucine or methionine at the 2nd major anchor position and valine at the last major anchor position (dominant anchor amino acids) and had high binding affinity to HLA-A2.1, the

isolated MART-1 or gp100 melanoma epitopes contain non-dominant amino acid at major anchor position such as alanine (the 2nd position of M9-2, the 9th position of G9-280) and threonine (the 2nd position of G9-154 and G9-209). The M9-2, G9-209 and G9-280 are not high affinity binders. By changing amino acid at the 1st, 2nd, 3rd or 9th positions which are important for HLA-A2 binding to the peptide, but less important for recognition by T-cell receptors, artificial peptides which can bind to HLA-A2.1 with higher affinity and still be recognized by natural epitope specific T-cells may be generated.

Among modified M9-2, G9-280, G9-209, G9-154 peptides, M9-2-2L, M9-2-1F, M9-2-3Y, G9-280-9V, G9-280-9L, G9-280-9I, G9-280-1F, G9-209-2L, G9-209-2M, G9-209-2I, G9-209-1F, G9-209-1Y, G9-209-1W2L, G9-209-1F2L, G9-209-1Y2L have higher binding affinity and were recognized by the original melanoma reactive T-cells. (Tables 14, 15, 16 and 17) PBL stimulated with autologous PBMC pulsed with G9-154-2I, G9-209-1F2L, or G9-280-9V (Tables 18, 19 and 20) recognized and lysed not only the original epitopes but also melanoma tumor cells (624mel) better than PBL stimulated with natural epitopes (G9-154, G9-209, G9-280).

These results demonstrated that modified peptides could be used for induction of anti-tumor T-cells instead of natural epitopes. Other peptides which were not recognized by the particular T-cells used in our study, but have higher binding affinity to HLA-A2.1 may induce a different set of T-cells capable of recognizing the original melanoma epitopes in <u>in vitro</u> or <u>in vivo</u>. These modified peptides may be used for induction of anti-melanoma T-cells in vitro and immunization of patients for the treatment of patients with melanoma or for the prevention of melanoma.

Table 14. Modified MART-1 M9-2 peptides

Peptide	Sequence		Binding affinity to HLA-A2.1 (nM)	Recognition by M9-2 reactive T-cell
M9-2 parent	AAGIGILTV	(SEQ ID NO:4)	1064	+
M9-2-2L	ALGIGILTV	(SEQ ID NO:50)	10	+
M9-2-2M	AMGIGILTV	(SEQ ID NO:51)	14	-
M9-2-2I	AIGIGILTV	(SEQ ID NO:52)	77	-
M9-2-1W	WAGIGILTV	(SEQ ID NO:53)	1351	+
M9-2-1F	FAGIGILTV	(SEQ ID NO:54)	244	+
M9-2-1Y	YAGIGILTV	(SEQ ID NO:55)	136	-
M9-2-3W	AAWIGILTV	(SEQ ID NO:56)	65	-
M9-2-3F	AAFIGILTV	(SEQ ID NO:57)	67	-
M9-2-3Y	AAYIGILTV	(SEQ ID NO:58)	102	+
M9-2-1K2L	KLGIGILTV	(SEQ ID NO:59)	14	-
M9-2-1K2M	KMGIGILTV	(SEQ ID NO:60)	27	-
M9-2-1K2I	KIGIGILTV	(SEQ ID NO:61)	94	-
M9-2-1W2L	WLGIGILTV	(SEQ ID NO:62)	11	-
M9-2-1F2L	FLGIGILTV	(SEQ ID NO:63)	1.8	-
M9-2-1Y2L	YLGIGILTV	(SEQ ID NO:64)	3.2	-
M9-2-2L3W	ALWIGILTV	(SEQ ID NO:65)	5.5	-
M9-2-2L3F	ALFIGILTV	(SEQ ID NO:66)	1.4	-
M9-2-2L3Y	ALYIGILTV	(SEQ ID NO:67)	3.7	-

Table 15. Modified gp100 G9-154 peptides

Peptide	Sequence		Binding affinity to HLA-A2.1 (nM)	Recognition by G9-154 reactive T-cell
G9-154 parent	KTWGQYWQV	(SEQ ID NO:46)	5.7	+
G9-154-2L	KLWGQYWQV	(SEQ ID NO:68)	2	+
G9-154-2M	KMWGQYWQV	(SEQ ID NO:69)	6.5	+
G9-154-2I	KIWGQYWQV	(SEQ ID NO:70)	3	+
G9-154-1W	WTWGQYWQV	(SEQ ID NO:71)	60	-
G9-154-1F	FTWGQYWQV	(SEQ ID NO:72)	1.6	-
G9-154-1Y	YTWGQYWQV	(SEQ ID NO:73)	2.5	-
G9-154-1A	ATWGQYWQV	(SEQ ID NO:74)	5.2	+
G9-154-1L	LTWGQYWQV	(SEQ ID NO:75)	3.4	+
G9-154-3Y	KTYGQYWQV	(SEQ ID NO:76)	30	+
G9-154-3F	KTFGQYWQV	(SEQ ID NO:77)	21	+
G9-154-1A2L	ALWGQYWQV	(SEQ ID NO:78)	2.3	+
G9-154-1L2L	LLWGQYWQV	(SEQ ID NO:79)	1.6	+
G9-154-1W2L	WLWGQYWQV	(SEQ ID NO:80)	2.8	-
G9-154-1F2L	FLWGQYWQV	(SEQ ID NO:81)	2.6	-
G9-154-1Y2L	YLWGQYWQV	(SEQ ID NO:82)	1.7	-

Table 16. Modified gp100 G9-209 peptides

Peptide	Sequence		Binding affinity to HLA-A2.1 (nM)*	Recognition by G9-209 reactive T-cell
G9-209 parent	ITDQVPFSV	(SEQ ID NO:48)	172	+
G9-209-2L	ILDQVPFSV	(SEQ ID NO:83)	3.3	+
G9-209-2M	IMDQVPFSV	(SEQ ID NO:84)	19	+
G9-209-2I	IIDQVPFSV	(SEQ ID NO:85)	40	+
G9-209-1F	FTDQVPFSV	(SEQ ID NO:86)	61	+
G9-209-1W	WTDQVPFSV	(SEQ ID NO:87)	711	+
G9-209-1Y	YTDQVPFSV	(SEQ ID NO:88)	85	+
G9-209-3W	ITWQVPFSV	(SEQ ID NO:89)	34	-
G9-209-3F	ITFQVPFSV	(SEQ ID NO:90)	66	-
G9-209-3Y	ITYQVPFSV	(SEQ ID NO:91)	33	-
G9-209-3A	ITAQVPFSV	(SEQ ID NO:92)	95	-
G9-209-3M	ITMQVPFSV	(SEQ ID NO:93)	40	-
G9-209-3S	ITSQVPFSV	(SEQ ID NO:94)	649	-
G9-209-2L3W	ILWQVPFSV	(SEQ ID NO:95)	1.7	-
G9-209-2L3F	ILFQVPFSV	(SEQ ID NO:96)	2	-
G9-209-2L3Y	ILYQVPFSV	(SEQ ID NO:97)	5	-
G9-209-2L3A	ILAQVPFSV	(SEQ ID NO:98)	11	-
G9-209-2L3M	ILMQVPFSV	(SEQ ID NO:99)	7.6	-
G9-209-2L3S	ILSQVPFSV	(SEQ ID NO:100)	20	-
G9-209-1W2L	WLDQVPFSV	(SEQ ID NO:101)	12	+
G9-209-1F2L	FLDQVPFSV	(SEQ ID NO:102)	2.2	+
G9-209-1Y2L	YLDQVPFSV	(SEQ ID NO:103)	2.3	+

a Concentration of sample peptide required for 50% inhibition of the standard radiolabeled peptide HBC18-27. Peptides are defined as high (50% inhibition at <50nM), intermediate (50-500nM) and weak (>500nM) binding peptides. (see example 4)

Table 17. Modified gp100 G9-280 peptides

Peptide	Sequence		Binding affinity to HLA-A2.1 (nM)	Recognition by G9-280 reactive T-cells
G9-280 parent	YLEPGPVTA	(SEQ ID NO:40)	455	+
G9-280-9V	YLEPGPVTV	(SEQ ID NO:104)	48	+
G9-280-9L	YLEPGPVTL	(SEQ ID NO:105)	88	+
G9-280-9I	YLEPGPVTI	(SEQ ID NO:106)	65	+
G9-280-1F	FLEPGPVTA	(SEQ ID NO:107)	125	+
G9-280-1W	WLEPGPVTA	(SEQ ID NO:108)	833	+
G9-280-3Y	YLYPGPVTA	(SEQ ID NO:109)	17	-
G9-280-3W	YLWPGPVTA	(SEQ ID NO:110)	3.2	-
G9-280-3F	YLFPGPVTA	(SEQ ID NO:111)	3.2	-
G9-280-3M	YLMPGPVTA	(SEQ ID NO:112)	4.3	-
G9-280-3S	YLSPGPVTA	(SEQ ID NO:113)	42	-
G9-280-3A	YLAPGPVTA	(SEQ ID NO:114)	9.3	-
G9-280-3M9V	YLMPGPVTV	(SEQ ID NO:115)	12	-
G9-280-3S9V	YLSPGPVTV	(SEQ ID NO:116)	23	-
G9-280-3A9V	YLAPGPVTV	(SEQ ID NO:117)	15	-
G9-280-3Y9V	YLYPGPVTV	(SEQ ID NO:118)	8.9	-
G9-280-3F9V	YLFPGPVTV	(SEQ ID NO:119)	5.8	-
G9-280-3W9V	YLWPGPVTV	(SEQ ID NO:120)	7.4	-

Table 18. Induction of anti-melanoma CTL using modified G9-154 peptide

Target	Effector T-cells: PBL stimulated with G9-154	Effector T-cells: PBL stimulated with G9-154-2I
	% specific lysis (E:T=40:1)	
T2	11	1
T2+G9-154	14	37
T2+G9-154-2I	8	38
624mel	5	23

^{51}Cr release assay was performed after 4 times stimulation with autologous PBMC preincubated with peptides.

Table 19. Induction of anti-melanoma CTL using modified G9-209 peptide

Target	Effector T-cells: PBL stimulated with G9-209	Effector T-cells: PBL stimulated with G9-209-1F2L
	% specific lysis (E:T=40:1)	
T2	0	0
T2+G9-209	6	85
T2+G9-209-1F2L	1	86
624mel	4	63

[51] Cr release assay was performed after 4 times stimulation with autologous PBMC preincubated with peptides.

Table 20. Induction of anti-melanoma CTL using modified G9-280 peptide

Target	Effector T-cells: PBL stimulated with G9-280	Effector T-cells: PBL stimulated with G9-280-9V
	% specific lysis (E:T=40:1)	
T2	3	0
T2+G9-280	11	87
T2+G9-280-9V	8	58
624mel	11	71

^{51}Cr release assay was performed after 4 times stimulation with autologous PBMC preincubated with peptides.

Example 6

MART-1 Vaccines As A Treatment For Melanoma In Mammals

MART-1 vaccines may be efficacious in treating mammals afflicted with melanoma. For example, MART-1 vaccines may be administered to individuals. Mammals can be immunized with the MART-1 proteins, peptides or modified peptides described herein in ranges of about 1mg- to about 100mg. Alternatively mammals, preferably humans may be immunized with the MART-1 nucleic acid sequence inserted into a viral vector such as vaccinia virus, adenovirus or fowl pox virus. A range of about 10^6 to about 10^{11} viral particles carrying the MART-1 nucleic acid sequences corresponding to immunogenic MART-1 peptides or modified peptides or analogs thereof, may be administered per mammal, preferably a human. The mammals will be monitored for antibodies to the immunogen or increase in cytotoxic lymphocytes (CTL) recognizing the immunogen by conventional methods or alleviation of clinical signs and symptoms of the active disease. Specific parameters to be assessed include production of immune cells that recognize the vaccine antigen or tumor regression. Such vaccines may be administered either prophylactically or therapeutically. Mammals may also be immunized with the gp-100 nucleic acid sequence inserted into a retroviral vector or GP-100 immunogenic peptides or modified peptides or analogs thereof. Suggested dose ranges of the antigen in retroviruses that may be used are about 10^6 to about 10^{11} viral particles per mammal, preferably a human. Response and efficacy of the retroviral vaccines will be assessed as described above.

Example 7

Use Of Lymphocytes Sensitized To Immunogenic Peptides Derived From Melanoma Antigens For Therapeutically Treating Mammals Afflicted With Melanoma

T-lymphocytes presensitized to the melanoma antigen

may be effective in therapeutically treating mammals afflicted with melanoma. The T-lymphocytes will be isolated from peripheral blood lymphocytes or tumor infiltrating lymphocytes and exposed in vitro to the MART-1 protein or peptide. T-lymphocytes are isolated from peripheral blood or melanoma tumor suspensions and cultured in vitro (Kawakami, Y. et al. (1988) J. Exp. Med. 168: 2183-2191). The T-lymphocytes are exposed to the MART-1 peptide AAGIGILTV for a period of about to 1-16 hours at a concentration of about 1 to about 10mg/ml. T-lymphocytes exposed to the antigen will be administered to the mammal, preferably a human at about 10^9 to about 10^{12} lymphocytes per mammal. Alternatively, the T-lymphocytes may be exposed to the modified MART-1 peptides. The lymphocytes may be administered either intravenously, intraperitoneally or intralesionally. This treatment may be administered concurrently with other therapeutic treatments such as cytokines, radiotherapy, surgical excision of melanoma lesions and chemotherapeutic drugs, adoptive T lymphocyte therapy. Alternatively, the T-lymphocytes may be exposed to the gp100 immunogenic peptides or modified immunogenic peptides described herein.

The present invention is not to be limited in scope by the nucleic acid sequences deposited, since the deposited embodiments is intended as a single illustration of one aspect of the invention and any sequences which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the dependent claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(F) POSTAL CODE: 20852

(ii) TITLE OF INVENTION: MELANOMA ANTIGENS AND THEIR USE IN DIAGNOSTIC AND THERAPEUTIC METHODS

(iii) NUMBER OF SEQUENCES: 126

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: FLOPPY DISK
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: ASCII

(vi) CURRENT APPLICATION DATA:
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(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US/08/231,565
(B) FILING DATE: 22-APR-1994
(C) CLASSIFICATION:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1559
(B) TYPE: NUCLEOTIDE
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGCAGACAGA GGACTCTCAT TAAGGAAGGT GTCCTGTGCC 40

CTGACCCTAC AAGATGCCAA GAGAAGATGC TCACTTCATC 80

TATGGTTACC CCAAGAAGGG GCACGGCCAC TCTTACACCA 120

CGGCTGAAGA GGCCGCTGGG ATCGGCATCC TGACAGTGAT 160

CCTGGGAGTC TTACTGCTCA TCGGCTGTTG GTATTGTAGA 200

AGACGAAATG GATACAGAGC CTTGATGGAT AAAAGTCTTC 240

ATGTTGGCAC TCAATGTGCC TTAACAAGAA GATGCCCACA 280

AGAAGGGTTT GATCATCGGG ACAGCAAAGT GTCTCTTCAA 320

GAGAAAAACT GTGAACCTGT GGTTCCCAAT GCTCCACCTG 360

CTTATGAGAA ACTCTCTGCA GAACAGTCAC CACCACCTTA 400

TTCACCTTAA GAGCCAGCGA GACACCTGAG ACATGCTGAA 440

ATTATTTCTC TCACACTTTT GCTTGAATTT AATACAGACA 480

TCTAATGTTC TCCTTTGGAA TGGTGTAGGA AAAATGCAAG 520

CCATCTCTAA TAATAAGTCA GTGTTAAAAT TTTAGTAGGT 560

CCGCTAGCAG TACTAATCAT GTGAGGAAAT GATGAGAAAT 600

ATTAAATTGG GAAAACTCCA TCAATAAATG TTGCAATGCA 640

TGATACTATC TGTGCCAGAG GTAATGTTAG TAAATCCATG 680

GTGTTATTTT CTGAGAGACA GAATTCAAGT GGGTATTCTG 720

GGGCCATCCA ATTTCTCTTT ACTTGAAATT TGGCTAATAA 760

CAAACTAGTC AGGTTTTCGA ACCTTGACCG ACATGAACTG 800

TACACAGAAT TGTTCCAGTA CTATGGAGTG CTCACAAAGG 840

ATACTTTTAC AGGTTAAGAC AAAGGGTTGA CTGGCCTATT 880

TATCTGATCA AGAACATGTC AGCAATGTCT CTTTGTGCTC 920

TAAAATTCTA TTATACTACA ATAATATATT GTAAAGATCC 960

TATAGCTCTT TTTTTTTGAG ATGGAGTTTC GCTTTTGTTG 1000

CCCAGGCTGG AGTGCAATGG CGCGATCTTG GCTCACCATA 1040

ACCTCCGCCT CCCAGGTTCA AGCAATTCTC CTGCCTTAGC 1080

CTCCTGAGTA GCTGGGATTA CAGGCGTGCG CCACTATGCC 1120

TGACTAATTT TGTAGTTTTA GTAGAGACGG GGTTTCTCCA 1160

TGTTGGTCAG GCTGGTCTCA AACTCCTGAC CTCAGGTGAT 1200

CTGCCCGCCT CAGCCTCCCA AAGTGCTGGA ATTACAGGCG 1240

TGAGCCACCA CGCCTGGCTG GATCCTATAT CTTAGGTAAG 1280

ACATATAACG CAGTCTAATT ACATTTCACT TCAAGGCTCA 1320

ATGCTATTCT AACTAATGAC AAGTATTTTC TACTAAACCA 1360

GAAATTGGTA GAAGGATTTA AATAAGTAAA AGCTACTATG 1400

TACTGCCTTA GTGCTGATGC CTGTGTACTG CCTTAAATGT 1440

ACCTATGGCA ATTTAGCTCT CTTGGGTTCC CAAATCCCTC 1480

TCACAAGAAT GTGCAGAAGA AATCATAAAG GATCAGAGAT 1520

TCTGAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA 1559

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 118
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Pro Arg Glu Asp Ala His Phe Ile Tyr Gly Tyr Pro Lys
 1               5                  10
Lys Gly His Gly His Ser Tyr Thr Thr Ala Glu Glu Ala Ala
15                  20                  25
Gly Ile Gly Ile Leu Thr Val Ile Leu Gly Val Leu Leu Leu
     30                  35                  40
Ile Gly Cys Trp Tyr Cys Arg Arg Arg Asn Gly Tyr Arg Ala
     45                  50                  55
Leu Met Asp Lys Ser Leu His Val Gly Thr Gln Cys Ala Leu
     60                  65                  70
Thr Arg Arg Cys Pro Gln Glu Gly Phe Asp His Arg Asp Ser
                 75                  80
Lys Val Ser Leu Gln Glu Lys Asn Cys Glu Pro Val Val Pro
 85                  90                  95
Asn Ala Pro Pro Ala Tyr Glu Lys Leu Ser Ala Glu Gln Ser
    100                 105                 110
Pro Pro Pro Tyr Ser Pro
        115
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Thr Thr Ala Glu Glu Ala Ala Gly Ile
 1               5
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Ala Ala Gly Ile Gly Ile Leu Thr Val
 1               5
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Ile Gly Ile Leu Thr Val Ile Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Ile Leu Thr Val Ile Leu Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ile Leu Thr Val Ile Leu Gly Val Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Leu Thr Val Ile Leu Gly Val Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Thr Val Ile Leu Gly Val Leu Leu Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Val Ile Leu Gly Val Leu Leu Leu Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9

(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ala Leu Met Asp Lys Ser Leu His Val
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ser Leu His Val Gly Thr Gln Cys Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Pro Val Val Pro Asn Ala Pro Pro Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Asn Ala Pro Pro Ala Tyr Glu Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Tyr Thr Thr Ala Glu Glu Ala Ala Gly Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Thr Ala Glu Glu Ala Ala Gly Ile Gly Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Glu Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Ala Ala Gly Ile Gly Ile Leu Thr Val Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Gly Ile Leu Thr Val Ile Leu Gly Val Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Ile Leu Thr Val Ile Leu Gly Val Leu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Leu Thr Val Ile Leu Gly Val Leu Leu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Thr Val Ile Leu Gly Val Leu Leu Leu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Arg Ala Leu Met Asp Lys Ser Leu His Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Ser Leu His Val Gly Thr Gln Cys Ala Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Ser Leu Gln Glu Lys Asn Cys Glu Pro Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2172
(B) TYPE: nucleotide
(C) STRANDEDNESS: Double
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTCGACGGCC ATTACCAATC GCGACCGGGA AGAACACAAT 40

GGATCTGGTG CTAAAAAGAT GCCTTCTTCA TTTGGCTGTG 80

ATAGGTGCTT TGCTGGCTGT GGGGGCTACA AAAGTACCCA 120

GAAACCAGGA CTGGCTTGGT GTCTCAAGGC AACTCAGAAC 160

CAAAGCCTGG AACAGGCAGC TGTATCCAGA GTGGACAGAA 200

GCCCAGAGAC TTGACTGCTG GAGAGGTGGT CAAGTGTCCC 240

TCAAGGTCAG TAATGATGGG CCTACACTGA TTGGTGCAAA 280

TGCCTCCTTC TCTATTGCCT TGAACTTCCC TGGAAGCCAA 320

AAGGTATTGC CAGATGGGCA GGTTATCTGG GTCAACAATA 360

CCATCATCAA TGGGAGCCAG GTGTGGGGAG GACAGCCAGT 400

GTATCCCCAG GAAACTGACG ATGCCTGCAT CTTCCCTGAT 440

GGTGGACCTT GCCCATCTGG CTCTTGGTCT CAGAAGAGAA 480

GCTTTGTTTA TGTCTGGAAG ACCTGGGGCC AATACTGGCA 520

ATTTCTAGGG GGCCCAGTGT CTGGGCTGAG CATTGGGACA 560

GGCAGGGCAA TGCTGGGCAC ACACACCATG GAAGTGACTG 600

TCTACCATCG CCGGGGATCC CGGAGCTATG TGCCTCTTGC 640

TCATTCCAGC TCAGCCTTCA CCATTACTGA CCAGGTGCCT 680

TTCTCCGTGA GCGTGTCCCA GTTGCGGGCC TTGGATGGAG 720

GGAACAAGCA CTTCCTGAGA AATCAGCCTC TGACCTTTGC 760

CCTCCAGCTC CATGACCCCA GTGGCTATCT GGCTGAAGCT 800

GACCTCTCCT ACACCTGGGA CTTTGGAGAC AGTAGTGGAA 840

CCCTGATCTC TCGGGCACTT GTGGTCACTC ATACTTACCT 880

GGAGCCTGGC CCAGTCACTG CCCAGGTGGT CCTGCAGGCT 920

GCCATTCCTC TCACCTCCTG TGGCTCCTCC CCAGTTCCAG 960

GCACCACAGA TGGGCACAGG CCAACTGCAG AGGCCCCTAA 1000

CACCACAGCT GGCCAAGTGC CTACTACAGA AGTTGTGGGT 1040

ACTACACCTG GTCAGGCGCC AACTGCAGAG CCCTCTGGAA 1080

CCACATCTGT GCAGGTGCCA ACCACTGAAG TCATAAGCAC 1120

TGCACCTGTG CAGATGCCAA CTGCAGAGAG CACAGGTATG 1160

ACACCTGAGA AGGTGCCAGT TTCAGAGGTC ATGGGTACCA 1200

CACTGGCAGA GATGTCAACT CCAGAGGCTA CAGGTATGAC 1240

ACCTGCAGAG GTATCAATTG TGGTGCTTTC TGGAACCACA 1280

GCTGCACAGG TAACAACTAC AGAGTGGGTG GAGACCACAG 1320

CTAGAGAGCT ACCTATCCCT GAGCCTGAAG GTCCAGATGC 1360

CAGCTCAATC ATGTCTACGG AAAGTATTAC AGGTTCCCTG 1400

GGCCCCCTGC TGGATGGTAC AGCCACCTTA AGGCTGGTGA 1440

AGAGACAAGT CCCCCTGGAT TGTGTTCTGT ATCGATATGG 1480

TTCCTTTTCC GTCACCCTGG ACATTGTCCA GGGTATTGAA 1520

AGTGCCGAGA TCCTGCAGGC TGTGCCGTCC GGTGAGGGGG 1560

ATGCATTTGA GCTGACTGTG TCCTGCCAAG GCGGGCTGCC 1600

CAAGGAAGCC TGCATGGAGA TCTCATCGCC AGGGTGCCAG 1640

CCCCCTGCCC AGCGGCTGTG CCAGCCTGTG CTACCCAGCC 1680

CAGCCTGCCA GCTGGTTCTG CACCAGATAC TGAAGGGTGG 1720

CTCGGGGACA TACTGCCTCA ATGTGTCTCT GGCTGATACC 1760

AACAGCCTGG CAGTGGTCAG CACCCAGCTT ATCATGCCTG 1800

GTCAAGAAGC AGGCCTTGGG CAGGTTCCGC TGATCGTGGG 1840

CATCTTGCTG GTGTTGATGG CTGTGGTCCT TGCATCTCTG 1880

ATATATAGGC GCAGACTTAT GAAGCAAGAC TTCTCCGTAC 1920

CCCAGTTGCC ACATAGCAGC AGTCACTGGC TGCGTCTACC 1960

CCGCATCTTC TGCTCTTGTC CCATTGGTGA GAACAGCCCC 2000

CTCCTCAGTG GGCAGCAGGT CTGAGTACTC TCATATGATG 2040

CTGTGATTTT CCTGGAGTTG ACAGAAACAC CTATATTTCC 2080

CCCAGTCTTC CCTGGGAGAC TACTATTAAC TGAAATAAAT 2120

ACTCAGAGCC TGAAAAAAAA TAAAAAAAAA AAAAAAAAAA 2160

AAAAAAAAAA AA 2172

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 661
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
Met Asp Leu Val Leu Lys Arg Cys Leu Leu His Leu
 1               5                  10
Ala Val Ile Gly Ala Leu Leu Ala Val Gly Ala Thr
            15                  20
Lys Val Pro Arg Asn Gln Asp Trp Leu Gly Val Ser
 25                  30                  35
Arg Gln Leu Arg Thr Lys Ala Trp Asn Arg Gln Leu
            40                  45
Tyr Pro Glu Trp Thr Glu Ala Gln Arg Leu Asp Cys
 50                  55                  60
Trp Arg Gly Gly Gln Val Ser Leu Lys Val Ser Asn
 65                  70
Asp Gly Pro Thr Leu Ile Gly Ala Asn Ala Ser Phe
            75                  80
Ser Ile Ala Leu Asn Phe Pro Gly Ser Gln Lys Val
 85                  90                  95
Leu Pro Asp Gly Gln Val Ile Trp Val Asn Asn Thr
            100                 105
Ile Ile Asn Gly Ser Gln Val Trp Gly Gly Gln Pro
        110                 115                 120
Val Tyr Pro Gln Glu Thr Asp Asp Ala Cys Ile Phe
                125                 130
Pro Asp Gly Gly Pro Cys Pro Ser Gly Ser Trp Ser
            135                 140
Gln Lys Arg Ser Phe Val Tyr Val Trp Lys Thr Trp
145                 150                 155
Gly Gln Tyr Trp Gln Phe Leu Gly Gly Pro Val Ser
                160                 165
Gly Leu Ser Ile Gly Thr Gly Arg Ala Met Leu Gly
        170                 175                 180
Thr His Thr Met Glu Val Thr Val Tyr His Arg Arg
                185                 190
Gly Ser Arg Ser Tyr Val Pro Leu Ala His Ser Ser
            195                 200
Ser Ala Phe Thr Ile Thr Asp Gln Val Pro Phe Ser
205                 210                 215
Val Ser Val Ser Gln Leu Arg Ala Leu Asp Gly Gly
                220                 225
Asn Lys His Phe Leu Arg Asn Gln Pro Leu Thr Phe
    230                 235                 240
```

Ala Leu Gln Leu His Asp Pro Ser Gly Tyr Leu Ala
245 250
Glu Ala Asp Leu Ser Tyr Thr Trp Asp Phe Gly Asp
255 260
Ser Ser Gly Thr Leu Ile Ser Arg Ala Leu Val Val
260 265 270
Thr His Thr Tyr Leu Glu Pro Gly Pro Val Thr Ala
275 280
Gln Val Val Leu Gln Ala Ala Ile Pro Leu Thr Ser
285 290 295
Cys Gly Ser Ser Pro Val Pro Gly Thr Thr Asp Gly
300 305
His Arg Pro Thr Ala Glu Ala Pro Asn Thr Thr Ala
310 315
Gly Gln Val Pro Thr Thr Glu Val Val Gly Thr Thr
320 325 330
Pro Gly Gln Ala Pro Thr Ala Glu Pro Ser Gly Thr
335 340
Thr Ser Val Gln Val Pro Thr Thr Glu Val Ile Ser
345 350 355
Thr Ala Pro Val Gln Met Pro Thr Ala Glu Ser Thr
360 365
Gly Met Thr Pro Glu Lys Val Pro Val Ser Glu Val
370 375
Met Gly Thr Thr Leu Ala Glu Met Ser Thr Pro Glu
380 385 390
Ala Thr Gly Met Thr Pro Ala Glu Val Ser Ile Val
395 400
Val Leu Ser Gly Thr Thr Ala Ala Gln Val Thr Thr
405 410 415
Thr Glu Trp Val Glu Thr Thr Ala Arg Glu Leu Pro
420 425
Ile Pro Glu Pro Glu Gly Pro Asp Ala Ser Ser Ile
430 435
Met Ser Thr Glu Ser Ile Thr Gly Ser Leu Gly Pro
440 445 450
Leu Leu Asp Gly Thr Ala Thr Leu Arg Leu Val Lys
455 460
Arg Gln Val Pro Leu Asp Cys Val Leu Tyr Arg Tyr
465 470 475
Gly Ser Phe Ser Val Thr Leu Asp Ile Val Gln Gly
480 490
Ile Glu Ser Ala Glu Ile Leu Gln Ala Val Pro Ser
495 500
Gly Glu Gly Asp Ala Phe Glu Leu Thr Val Ser Cys
505 510 515
Gln Gly Gly Leu Pro Lys Glu Ala Cys Met Glu Ile
520 525
Ser Ser Pro Gly Cys Gln Pro Pro Ala Gln Arg Leu
530 535 540
Cys Gln Pro Val Leu Pro Ser Pro Ala Cys Gln Leu
545 550
Val Leu His Gln Ile Leu Lys Gly Gly Ser Gly Thr
555 560

```
Tyr Cys Leu Asn Val Ser Leu Ala Asp Thr Asn Ser
565                 570                 575
Leu Ala Val Val Ser Thr Gln Leu Ile Met Pro Gly
            580                 585
Gln Glu Ala Gly Leu Gly Gln Val Pro Leu Ile Val
    590                 595                 600
Gly Ile Leu Leu Val Leu Met Ala Val Val Leu Ala
                605                 610
Ser Leu Ile Tyr Arg Arg Arg Leu Met Lys Gln Asp
        615                 620
Phe Ser Val Pro Gln Leu Pro His Ser Ser Ser His
625                 630                 635
Trp Leu Arg Leu Pro Arg Ile Phe Cys Ser Cys Pro
            640                 645
Ile Gly Glu Asn Ser Pro Leu Leu Ser Gly Gln Gln
    650                 655                 660
Val
```

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

```
Val Pro Gly Ile Leu Leu Thr
 1               5
```

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
Leu Leu Ser Gly Gln Gln Val
 1               5
```

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Pro Pro Gln Trp Ala Ala Gly Leu Ser Thr Leu Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Met Leu Leu Ala Val Leu Tyr Cys Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Tyr Met Asn Gly Thr Met Ser Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid

(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Leu Leu Asp Gly Thr Ala Thr Leu Arg Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

Val Leu Tyr Arg Tyr Gly Ser Phe Ser Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

Ala Leu Asp Gly Gly Asn Lys His Phe Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

Val Leu Lys Arg Cys Leu Leu His Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

Val Leu Pro Ser Pro Ala Cys Gln Leu Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

Ser Leu Ala Asp Thr Asn Ser Leu Ala Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

Ser Val Ser Val Ser Gln Leu Arg Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

Tyr Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

Leu Asn Val Ser Leu Ala Asp Thr Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58
(B) TYPE: NUCLEOTIDE
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

GGACAGGCCG AGGCGGCCTT TTTTTTTTTT TTTTTTTTTT 40
TTTTTTTTTT TTTTTTTT 58

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12
(B) TYPE: NUCLEOTIDE
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

CCAATCGCGA CC 12

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14
(B) TYPE: NUCLEOTIDE
(C) STRANDEDNESS: DOUBLE
(D) TOPOLOGY: UNKNOWN

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

GGTCGCGATTG GTAA 14

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

Gly Ile Leu Gly Phe Val Phe Thr Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

Lys Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

Lys Thr Trp Gly Gln Tyr Trp Gln Val Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

Ile Thr Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

Thr Ile Thr Asp Gln Val Pro Phe Ser Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

Ala Leu Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

Ala Met Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

Ala Ile Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

Trp Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9

(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

Phe Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

Tyr Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

Ala Ala Trp Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

Ala Ala Phe Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

Ala Ala Tyr Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

Lys Leu Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

Lys Met Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

Lys Ile Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

Trp Leu Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

Phe Leu Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 64:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

Tyr Leu Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

Ala Leu Trp Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

Ala Leu Phe Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

Ala Leu Tyr Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

Lys Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

Lys Met Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

Lys Ile Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

Trp Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9

(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

Phe Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

Tyr Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

Ala Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

Leu Thr Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

Lys Thr Tyr Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

Lys Thr Phe Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

Ala Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

Leu Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

Trp Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

Phe Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

Tyr Leu Trp Gly Gln Tyr Trp Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

Ile Leu Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

Ile Met Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

Ile Ile Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

Phe Thr Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

Trp Thr Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

Tyr Thr Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

Ile Thr Trp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9

(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

Ile Thr Phe Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

Ile Thr Tyr Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

Ile Thr Ala Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

Ile Thr Met Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:

Ile Thr Ser Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

Ile Leu Trp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 96:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 96:

Ile Leu Phe Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 97:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 97:

Ile Leu Tyr Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98:

Ile Leu Ala Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 99:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 99:

Ile Leu Met Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 100:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 100:

Ile Leu Ser Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 101:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 101:

Trp Leu Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 102:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 102:

Phe Leu Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 103:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 103:

Tyr Leu Asp Gln Val Pro Phe Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO: 104:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 104:

Tyr Leu Glu Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 105:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 105:

Tyr Leu Glu Pro Gly Pro Val Thr Leu
1 5

(2) INFORMATION FOR SEQ ID NO: 106:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 106:

Tyr Leu Glu Pro Gly Pro Val Thr Ile
1 5

(2) INFORMATION FOR SEQ ID NO: 107:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 107:

Phe Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 108:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9

(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 108:

Trp Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 109:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 109:

Tyr Leu Tyr Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 110:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 110:

Tyr Leu Trp Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 111:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 111:

Tyr Leu Phe Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 112:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 112:

Tyr Leu Met Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 113:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 113:

Tyr Leu Ser Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 114:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 114:

Tyr Leu Ala Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO: 115:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 115:

Tyr Leu Met Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 116:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 116:

Tyr Leu Ser Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 117:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 117:

Tyr Leu Ala Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 118:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 118:

Tyr Leu Tyr Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 119:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 119:

Tyr Leu Phe Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 120:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 120:

Tyr Leu Trp Pro Gly Pro Val Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO: 121:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 661
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 121:

Met Asp Leu Val Leu Lys Arg Cys Leu Leu His Leu
1 5 10
Ala Val Ile Gly Ala Leu Leu Ala Val Gly Ala Thr
15 20
Lys Val Pro Arg Asn Gln Asp Trp Leu Gly Val Ser
25 30 35
Arg Gln Leu Arg Thr Lys Ala Trp Asn Arg Gln Leu
40 45
Tyr Pro Glu Trp Thr Glu Ala Gln Arg Leu Asp Cys
50 55 60

Trp Arg Gly Gly Gln Val Ser Leu Lys Val Ser Asn
65 70
Asp Gly Pro Thr Leu Ile Gly Ala Asn Ala Ser Phe
75 80
Ser Ile Ala Leu Asn Phe Pro Gly Ser Gln Lys Val
85 90 95
Leu Pro Asp Gly Gln Val Ile Trp Val Asn Asn Thr
100 105
Ile Ile Asn Gly Ser Gln Val Trp Gly Gly Gln Pro
110 115 120
Val Tyr Pro Gln Glu Thr Asp Asp Ala Cys Ile Phe
125 130
Pro Asp Gly Gly Pro Cys Pro Ser Gly Ser Trp Ser
135 140
Gln Lys Arg Ser Phe Val Tyr Val Trp Lys Thr Trp
145 150 155
Gly Gln Tyr Trp Gln Val Leu Gly Gly Pro Val Ser
160 165
Gly Leu Ser Ile Gly Thr Gly Arg Ala Met Leu Gly
170 175 180
Thr His Thr Met Glu Val Thr Val Tyr His Arg Arg
185 190
Gly Ser Arg Ser Tyr Val Pro Leu Ala His Ser Ser
195 200
Ser Ala Phe Thr Ile Thr Asp Gln Val Pro Phe Ser
205 210 215
Val Ser Val Ser Gln Leu Arg Ala Leu Asp Gly Gly
220 225
Asn Lys His Phe Leu Arg Asn Gln Pro Leu Thr Phe
230 235 240
Ala Leu Gln Leu His Asp Pro Ser Gly Tyr Leu Ala
245 250
Glu Ala Asp Leu Ser Tyr Thr Trp Asp Phe Gly Asp
255 260
Ser Ser Gly Thr Leu Ile Ser Arg Ala Leu Val Val
260 265 270
Thr His Thr Tyr Leu Glu Pro Gly Pro Val Thr Ala
275 280
Gln Val Val Leu Gln Ala Ala Ile Pro Leu Thr Ser
285 290 295
Cys Gly Ser Ser Pro Val Pro Gly Thr Thr Asp Gly
300 305
His Arg Pro Thr Ala Glu Ala Pro Asn Thr Thr Ala
310 315
Gly Gln Val Pro Thr Thr Glu Val Val Gly Thr Thr
320 325 330
Pro Gly Gln Ala Pro Thr Ala Glu Pro Ser Gly Thr
335 340
Thr Ser Val Gln Val Pro Thr Thr Glu Val Ile Ser
345 350 355
Thr Ala Pro Val Gln Met Pro Thr Ala Glu Ser Thr
360 365
Gly Met Thr Pro Glu Lys Val Pro Val Ser Glu Val
370 375

Met Gly Thr Thr Leu Ala Glu Met Ser Thr Pro Glu
380 385 390
Ala Thr Gly Met Thr Pro Ala Glu Val Ser Ile Val
395 400
Val Leu Ser Gly Thr Thr Ala Ala Gln Val Thr Thr
405 410 415
Thr Glu Trp Val Glu Thr Thr Ala Arg Glu Leu Pro
420 425
Ile Pro Glu Pro Glu Gly Pro Asp Ala Ser Ser Ile
430 435
Met Ser Thr Glu Ser Ile Thr Gly Ser Leu Gly Pro
440 445 450
Leu Leu Asp Gly Thr Ala Thr Leu Arg Leu Val Lys
455 460
Arg Gln Val Pro Leu Asp Cys Val Leu Tyr Arg Tyr
465 470 475
Gly Ser Phe Ser Val Thr Leu Asp Ile Val Gln Gly
480 490
Ile Glu Ser Ala Glu Ile Leu Gln Ala Val Pro Ser
495 500
Gly Glu Gly Asp Ala Phe Glu Leu Thr Val Ser Cys
505 510 515
Gln Gly Gly Leu Pro Lys Glu Ala Cys Met Glu Ile
520 525
Ser Ser Pro Gly Cys Gln Pro Pro Ala Gln Arg Leu
530 535 540
Cys Gln Pro Val Leu Pro Ser Pro Ala Cys Gln Leu
545 550
Val Leu His Gln Ile Leu Lys Gly Gly Ser Gly Thr
555 560
Tyr Cys Leu Asn Val Ser Leu Ala Asp Thr Asn Ser
565 570 575
Leu Ala Val Val Ser Thr Gln Leu Ile Met Pro Gly
580 585
Gln Glu Ala Gly Leu Gly Gln Val Pro Leu Ile Val
590 595 600
Gly Ile Leu Leu Val Leu Met Ala Val Val Leu Ala
605 610
Ser Leu Ile Tyr Arg Arg Arg Leu Met Lys Gln Asp
615 620
Phe Ser Val Pro Gln Leu Pro His Ser Ser Ser His
625 630 635
Trp Leu Arg Leu Pro Arg Ile Phe Cys Ser Cys Pro
640 645
Ile Gly Glu Asn Ser Pro Leu Leu Ser Gly Gln Gln
650 655 660
Val

(2) INFORMATION FOR SEQ ID NO: 122:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid

(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 122:

Xaa Xaa Xaa Ile Gly Ile Leu Thr Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 123:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 123:

Xaa Xaa Xaa Gly Gln Tyr Trp Gln Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 124:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 124:

Xaa Xaa Xaa Gln Val Pro Phe Ser Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 125:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 125:

Xaa Xaa Xaa Pro Gly Pro Val Thr Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 126:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10
(B) TYPE: amino acid
(C) STRANDEDNESS: Unknown
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 126:

Phe Leu Pro Ser Asp Tyr Phe Pro Ser Val
1 5 10

CLAIMS:

1. An isolated nucleic acid sequence encoding MART-1.

2. The nucleic acid sequence of claim 1 having the sequence shown in Figure 1 (SEQ ID NO: 1).

3. The nucleic acid sequence of claim 1 wherein said sequence is an allelic variation of the sequence shown in Figure 1 (SEQ ID NO: 1).

4. The nucleic acid sequence of claim 1 wherein said sequence is a homolog of the sequence shown in Figure 1 (SEQ ID NO: 1).

5. The nucleic acid sequence of claim 1 wherein said sequence is a variant of the sequence in Figure 1 (SEQ ID NO: 1).

6. A recombinant protein encoded by the nucleic acid sequence of claim 1.

7. A recombinant protein encoded by the nucleic acid sequence of claim 2.

8. A recombinant protein encoded by the nucleic acid sequence of claim 3.

9. A recombinant protein encoded by the nucleic acid sequence of claim 4.

10. A recombinant protein encoded by the nucleic acid sequence of claim 5.

11. An isolated and purified protein comprising the amino acid sequence shown in Figure 1 (SEQ ID NO: 2) or a substantially homologous sequence thereof.

12. A peptide having the sequence AAGIGILTV (SEQ ID NO: 4), EAAGIGILTV (SEQ ID NO: 17), or AAGIGILTVI (SEQ ID NO: 18).

13. A method of producing the recombinant protein, according to claim 1, comprising:

(a) inserting the nucleic acid sequence shown in Figure 1 (SEQ ID NO: 1) sequence into an expression vector;

(b) transferring the expression vector into a host cell;

(c) culturing the host organism under conditions appropriate for amplification of the vector and expression of the protein; and

(d) harvesting the protein.

14. The method of claim 13, wherein the expression vector is a eukaryotic expression vector or prokaryotic expression vector.

15. The method of claim 13, wherein the expression vector is a baculovirus vector.

16. The method of claim 13, wherein the host cell is a eukaryotic cell or prokaryotic cell.

17. The method of claim 13, wherein the eukaryotic cell is an insect cell.

18. A recombinant expression vector comprising all or part of the nucleic acid sequence of claim 1.

19. A host organism transformed or transfected with the recombinant expression vector according to claim 18 in a manner to allow expression of said protein encoded by said recombinant expression vector.

20. Antibodies reactive with the protein according to claim 11 or portions thereof.

21. The antibodies of claim 20 wherein said antibodies are monoclonal.

22. The antibodies of claim 20 wherein said antibodies are polyclonal.

23. A method for detecting MART-1 messenger RNA in a biological sample comprising the steps of:

(a) contacting all or part of the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) with said biological sample under conditions allowing a complex to form between said nucleic acid sequence and said messenger RNA
(b) detecting said complexes; and
(c) determining the level of said messenger RNA.

24. The method of claim 23 wherein said sample is selected from the group consisting of mammalian tissues, mammalian cells, necropsy samples, pathology samples and biopsy samples.

25. The method of claim 24 wherein said biological sample is from a mammal afflicted with a disease state.

26. The method of claim 25 wherein said determination of said level of said mRNA is used to diagnose, assess or prognose the disease state.

27. The method of claim 26 wherein said biological sample is from a mammal afflicted with melanoma or metastatic melanoma.

28. A method of detecting MART-1 protein in a biological sample comprising the steps of:

(a) contacting a reagent which specifically reacts and forms a complex with said protein in said sample; and

(b) detecting the formation of said complex between said protein and said reagent.

29. The method of claim 28 wherein said sample is selected from the group consisting of mammalian tissues, mammalian cells, necropsy samples, pathology samples, and biopsy samples.

30. The method of claim 28 wherein said reagent is an antibody or fragment thereof.

31. The method of claim 28 wherein said reagent is monoclonal antibody.

32. The method of claim 28 wherein said reagent is a polyclonal antibody.

33. The method of claim 28 wherein said biological sample is from a mammal afflicted with a disease state.

34. The method of claim 28 wherein said determination of said level of said protein is used to diagnose, assess or prognose the disease state.

35. The method of claim 34 wherein said disease state is melanoma or metastatic melanoma.

36. A method of detecting MART-1 genomic nucleic acid sequences in a biological sample comprising the steps of:

(a) contacting all or part of the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) with a biological sample under condition to allow complexes to form between said nucleic acid sequence and said genomic DNA sequences; and

(b) determining alterations in said genomic sequence.

37. The method of claim 36 wherein said alteration is a deletion, substitution, addition or amplification of said genomic DNA sequences.

38. An immunogenic peptide having contiguous amino acids derived from the MART-1 sequence (SEQ ID NO: 2) or analogs thereof.

39. The immunogenic peptides of claim 38 wherein such peptides are at least about 9 to 10 amino acids in length.

40. The immunogenic peptide of claim 38 where said peptide has the sequence selected from the group consisting of (i) AAGIGILTV (SEQ ID NO: 4), (ii) EAAGIGILTV (SEQ ID NO: 17), (iii) AAGIGILTVI (SEQ ID NO: 18) and an analog of any one (i) - (iii).

41. The peptide of claim 41, wherein said peptide is AAGIGILTV.

42. The immunogenic peptide of claim 38, wherein said peptide sequence contains at least one amino acid modification of said MART-1 sequence to enhance binding of peptide to an MHC molecule.

43. The immunogenic peptides of claim 42 wherein such

peptides are at least about 9 to 10 amino acids in length.

44. The peptide of claim 42, wherein said modification includes at least one amino acid substitution in said peptide sequence.

45. The peptide of claim 44, wherein said amino acid substitution is located at a position selected from the group consisting of: (i) the first position, (ii) the second position, (iii) the third position, (iv) the ninth position, (v) tenth position and (vi) combinations of at least two of (i) - (v) in the sequence of the peptide.

46. The peptide of claim 45, wherein said amino acid substitution are located at the second and ninth positions.

47. An immunogenic peptide having the formula $X_1X_2X_3IGILTX_4$ wherein:

X_1 may be any amino acid;
X_2 may be any hydrophobic aliphatic amino acid;
X_3 may be any amino acid; and
X_4 may be any hydrophobic aliphatic amino acid.

48. The peptide of claim 47 wherein X_1 is selected from the group consisting of methionine, leucine, alanine, glycine, threonine, isoleucine, tyrosine, valine, tryptophan, phenylalanine, serine, lysine or aspartic acid.

49. The peptide of claim 47 wherein X_2 is selected from the group consisting of methionine, leucine, alanine, glycine, isoleucine, valine or threonine.

50. The peptide of claim 47 wherein X_3 is selected from the group consisting of methionine, leucine, alanine,

glycine, threonine, isoleucine, tyrosine, valine, tryptophan, phenylalanine, lysine, serine or aspartic acid.

51. The peptide of claim 47 wherein X_4 is selected from the group consisting of methionine, leucine, alanine, glycine, isoleucine, valine or threonine.

52. The peptide of claim 47 wherein said peptide has a sequence shown in Table 14.

53. An immunogenic peptide having contiguous amino acids derived from a gp100 sequence.

54. The immunogenic peptide of claim 53 wherein said peptide is at least about 9 to 10 amino acids in length.

55. The immunogenic peptide of claim 54 wherein the peptide is selected from the group consisting of LLDGTATLRL (SEQ ID NO: 33), VLYRYGSFSV (SEQ ID NO: 34), VLKRCLLHL (SEQ ID NO: 36), ALDGGNKHFL (SEQ ID NO: 35), VLPSPACQLV (SEQ ID NO: 37), YLEPGPVTA (SEQ ID NO: 40), SLADTNSLAV (SEQ ID NO: 38), SVSVSQLRA (SEQ ID NO:39), LNVSLADTN (SEQ ID NO:41), KTWGQYWQV (SEQ ID NO:46), KTWGQYWQVL (SEQ ID NO:47), ITDQVPFSV (SEQ ID NO:48) and TITDQVPFSV (SEQ ID NO:49).

56. The immunogenic peptide of claims 53, 54 or 55 wherein said peptide contains at least one amino acid modification of said gp100 sequence.

57. The peptide of claim 56, wherein said modification includes at least one amino acid substitution in said peptide sequence.

58. The peptide of claim 56, wherein said amino acid

substitution is located at a position selected from the group consisting of: (i) the first position, (ii) the second position, (iii) the third position, (iv) the ninth position, (v) tenth position and (vi) combinations of at least two of (i) - (v) in the sequence of the peptide.

59. An immunogenic peptide having the a formula selected from the group consisting of $X_1X_2X_3GQYWQX_4$, $X_1X_2X_3QVPFSX_4$ and $X_1X_2X_3PGPVTX_4$ wherein:

X_1 is any amino acid;

X_2 is any hydrophobic aliphatic amino acid;

X_3 is any amino acid; and

X_4 is a hydrophobic aliphatic amino acid.

60. The peptide of claim 59 wherein the amino acid for X_1 is selected from the group consisting of methionine, leucine, alanine, glycine, threonine, isoleucine, valine tyrosine, serine, tryptophan, phenylalanine, serine, lysine or aspartic acid.

61. The peptide of claim 59 wherein X_2 is selected from the group consisting of methionine, leucine, alanine, glycine, isoleucine, valine or threonine.

62. The peptide of claim 59 wherein X_3 is selected from the group consisting of methionine, leucine, alanine, glycine, threonine, isoleucine, tyrosine, valine, tryptophan, phenylalanine, serine, lysine or aspartic acid.

63. The peptide of claim 59 wherein X_4 is selected from the group consisting of methionine, leucine, alanine, glycine, isoleucine, valine or threonine.

64. The immunogenic peptide of any of claims 38, 47, 53 or 59 wherein said peptide is recognized by HLA-A2

restricted tumor infiltrating lymphocyte.

65. The immunogenic peptide of any one of claims 38, 47, 53 or 59 wherein said peptide is a native, synthetic or recombinant peptide.

66. A pharmaceutical composition comprising the recombinant proteins of claim 6 and an acceptable excipient, diluent or carrier.

67. A method of preventing or treating melanoma comprising administering the pharmaceutical composition of claim 66 to a mammal in an effective amount to stimulate the production of protective antibodies or immune cells.

68. A vaccine for immunizing a mammal comprising a recombinant protein according to claim 66 in a pharmacologically acceptable carrier.

69. A pharmaceutical composition comprising the peptides of any one of claims 38, 47, 53 or 59 and an acceptable excipient, diluent or carrier.

70. A method of preventing or treating melanoma comprising administering the pharmaceutical composition of claim 69 to a mammal in an effective amount to stimulate the production of protective antibodies or immune cells.

71. A vaccine for immunizing a mammal comprising a peptide according to any one of claims 38, 47, 53 or 59 in a pharmacologically acceptable carrier.

72. A purified and isolated nucleic acid sequence encoding a peptide according to any one of claims 38, 47, 53 or 59.

73. A recombinant expression vector comprising at least one nucleic acid sequence of any one of claim 73.

74. The vector of claim 73, wherein the expression vector is a eukaryotic expression vector or prokaryotic expression vector.

75. A host organism transformed or transfected with the recombinant expression vector according to claim 74 in a manner to allow expression of said protein encoded by said recombinant expression vector.

76. Antibodies reactive with the immunogenic peptide according to claims 38, 47, 53 or 59.

77. The antibodies of claim 76 wherein said antibodies are monoclonal.

78. The antibodies of claim 76 wherein said antibodies are polyclonal.

79. A method of identifying genes encoding melanoma antigens using tumor infiltrating lymphocytes (TIL), said method comprising the following steps:

(a) isolating tumor infiltrating lymphocytes from a tumor from a mammal afflicted with melanoma;

(b) introducing a melanoma cDNA library into a mammalian cell line;

(c) exposing said mammalian cells (from step 5) to said TIL;

(d) screening for expression of an antigen encoded by said cDNA in said mammalian cells recognized by said TIL; and

(e) isolating said cDNA corresponding to said antigen.

80. The method of claim 79 wherein said cells in step (b) are selected from the group consisting of tumor cell lines or COS 7 cells.

81. A method for assessing immunogenicity of peptides derived from amino acid sequences of a MART-1 protein having the sequence (Figure 1; SEQ ID NO: 2) or a gp100 protein having the sequence (Figure 5A; SEQ ID NO: 27 or Figure 71; (SEQ ID NO:121) said method comprising the steps of:

(a) preparing a plurality of peptides based on the MART-1 or gp100 amino acid sequence;

(b) incubating at least one of said peptides with a mammalian cell line;

(c) exposing said mammalian cells incubated with said peptide to tumor infiltrating lymphocytes (TIL); and

(d) screening for recognition of TIL with said cells incubated with said peptide.

82. The method of claims 81 wherein said peptides in step (a) are about 9 to 10 amino acids.

83. The method of claim 81 wherein said cells in step (b) are selected from the group of COS cells, T2 cells, or EBV transformed B cell lines.

84. A purified and isolated nucleic acid sequence encoding a peptide comprising at least about 8 contiguous amino acids, said peptide being derived from the MART-1 sequence (Figure 1; SEQ ID NO: 2) or a gp100 sequence said peptide being reactive to tumor infiltrating lymphocytes (TIL).

85. A recombinant expression vector comprising at least one nucleic acid sequence of claim 84.

86. The use of the immunogenic peptides of claims 38, 47, 53, or 59 in the manufacture of a medicament.

87. The use of the immunogenic peptides of claims 38, 47, 53, or 59 in the treatment or prevention of melanoma.

88. The use of the recombinant expression vectors of claims 10, 73, or 84 in the manufacture of a medicament.

89. The use of the recombinant expression vectors of claims 18, 73, or 84 in the treatment or prevention of melanoma.

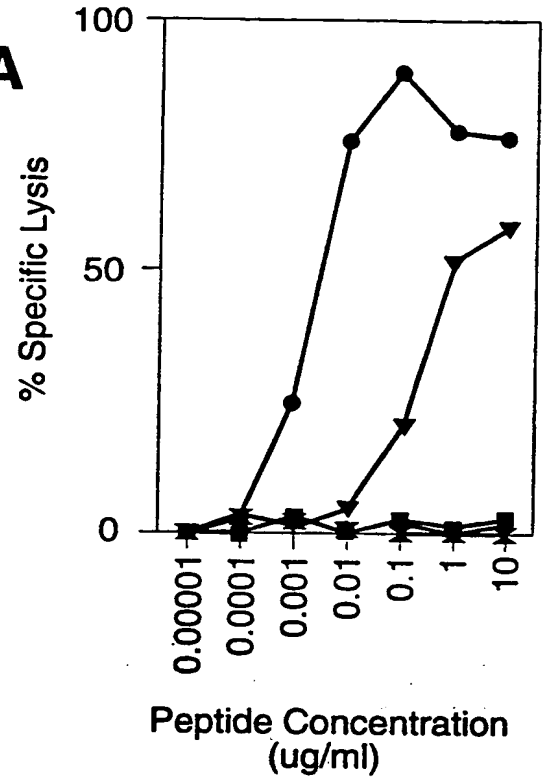
90. The use of the nucleic acid sequences of claims 1 to 73 in the manufacture of a medicament.

91. The use of the nucleic and sequences of claims 1 or 73 in the treatment or prevention of melanoma.

FIG. 1

```
1     AGCAGACAGAGGACTCTCATTAAGGAAGG TGTCCTGTGCCCTGACCCTACAAGATGCCA  59
                                                        MetPro
60    AGAGAAGATGCTCACTTCATCTATGGTTAC CCCAAGAAGGGGCACGGCCACTCTTACACC 119
3     ArgGluAspAlaHisPheIleTyrGlyTyr ProLysLysGlyHisGlyHisSerTyrThr  22

120   ACGGCTGAAGAGGCCGCTGGGATCGGCATC CTGACAGTGATCCTGGGAGTCTTACTGCTC 179
23    ThrAlaGluGluAlaAlaGlyIleGlyIle LeuThrValIleLeuGlyValLeuLeuLeu  42

180   ATCGGCTGTTGGTATTGTAGAAGACGAAAT GGATACAGAGCCTTGATGGATAAAAGTCTT 239
43    IleGlyCysTrpTyrCysArgArgArgAsn GlyTyrArgAlaLeuMetAspLysSerLeu  62

240   CATGTTGGCACTCAATGTGCCTTAACAAGA AGATGCCCACAAGAAGGGTTTGATCATCGG 299
63    HisValGlyThrGlnCysAlaLeuThrArg ArgCysProGlnGluGlyPheAspHisArg  82

300   GACAGCAAAGTGTCTCTTCAAGAGAAAAAC TGTGAACCTGTGGTTCCCAATGCTCCACCT 359
83    AspSerLysValSerLeuGlnGluLysAsn CysGluProValValProAsnAlaProPro 102

360   GCTTATGAGAAACTCTCTGCAGAACAGTCA CCACCACCTTATTCACCTTAAGAGCCAGCG 419
103   AlaTyrGluLysLeuSerAlaGluGlnSer ProProProTyrSerPro

420   AGACACCTGAGACATGCTGAAATTATTTCT CTCACACTTTGCTTGAATTTAATACAGAC 479

480   ATCTAATGTTCTCCTTTGGAATGGTGTAGG AAAAATGCAAGCCATCTCTAATAATAAGTC 539
540   AGTGTTAAAATTTTAGTAGGTCCGCTAGCA GTACTAATCATGTGAGGAAATGATGAGAAA 599
600   TATTAAATTGGGAAAACTCCATCAATAAAT GTTGCAATGCATGATACTATCTGTGCCAGA 659
660   GGTAATGTTAGTAAATCCATGGTGTTATTT TCTGAGAGACAGAATTCAAGTGGGTATTCT 719
720   GGGGCCATCCAATTTCTCTTTACTTGAAAT TTGGCTAATAACAAACTAGTCAGGTTTTCG 779
780   AACCTTGACCGACATGAACTGTACACAGAA TTGTTCCAGTACTATGGAGTGCTCACAAAG 839
840   GATACTTTTACAGGTTAAGACAAAGGGTTG ACTGGCCTATTTATCTGATCAAGAACATGT 899
900   CAGCAATGTCTCTTTGTGCTCTAAAATTCT ATTATACTACAATAATATTGTAAAGATC 959
960   CTATAGCTCTTTTTTTTTTGAGATGGAGTTT CGCTTTTGTTGCCCAGGCTGGAGTGCAATG 1019
1020  GCGCGATCTTGGCTCACCATAACCTCCGCC TCCCAGGTTCAAGCAATTCTCCTGCCTTAG 1079
1080  CCTCCTGAGTAGCTGGGATTACAGGCGTGC GCCACTATGCCTGACTAATTTTTGTAGTTTT 1139
1140  AGTAGAGACGGGGTTTCTCCATGTTGGTCA GGCTGGTCTCAAACTCCTGACCTCAGGTGA 1199
1200  TCTGCCCGCCTCAGCCTCCCAAAGTGCTGG AATTACAGGCGTGAGCCACCACGCCTGGCT 1259
1260  GGATCCTATATCTTAGGTAAGACATATAAC GCAGTCTAATTTACATTTCACTTCAAGGCTC 1319
1320  AATGCTATTCTAACTAATGACAAGTATTTT CTACTAAACCAGAAATTGGTAGAAGGATTT 1379
1380  AAATAAGTAAAAGCTACTATGTACTGCCTT AGTGCTGATGCCTGTGTACTGCCTTAAATG 1439
1440  TACCTATGGCAATTTAGCTCTCTTGGGTTC CCAAATCCCTCTCACAAGAATGTGCAGAAG 1499
1500  AAATCATAAAGGATCAGAGATTCTGAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1559
```

FIG. 2A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0.0001
0.001
0.01
0.1
1
10
Peptide Concentration
(ug/ml)

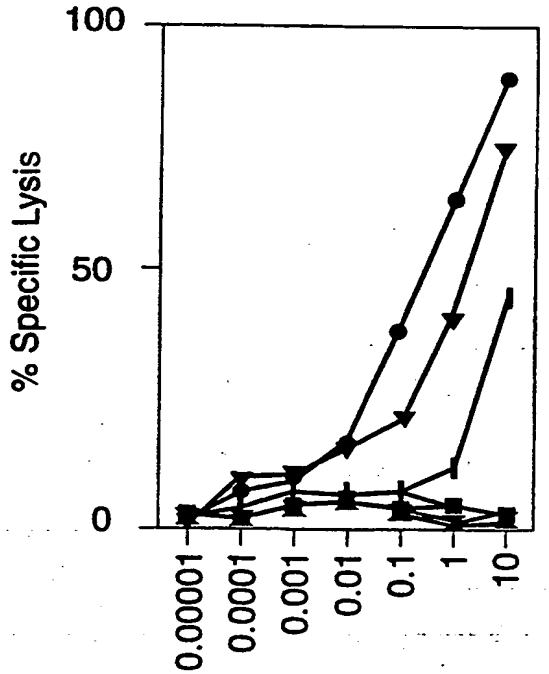

100
50
0
% Specific Lysis
0.00001
0.0001
0.001
0.01
0.1
1
10
Peptide Concentration
(ug/ml)

FIG. 2B

FIG. 3A

FIG. 3B

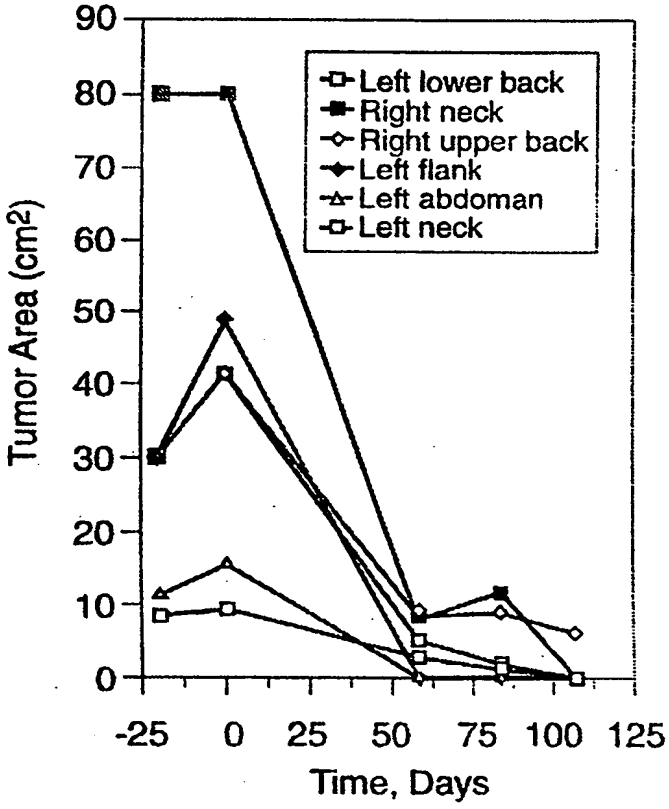


FIG. 4A

```
GTCGACGGCC ATTACCAATC GCGACCGGGA AGAACACAAT    40
GGATCTGGTG CTAAAAAGAT GCCTTCTTCA TTTGGCTGTG    80
ATAGGTGCTT TGCTGGCTGT GGGGGCTACA AAAGTACCCA   120
GAAACCAGGA CTGGCTTGGT GTCTCAAGGC AACTCAGAAC   160
CAAAGCCTGG AACAGGCAGC TGTATCCAGA GTGGACAGAA   200
GCCCAGAGAC TTGACTGCTG GAGAGGTGGT CAAGTGTCCC   240
TCAAGGTCAG TAATGATGGG CCTACACTGA TTGGTGCAAA   280
TGCCTCCTTC TCTATTGCCT TGAACTTCCC TGGAAGCCAA   320
AAGGTATTGC CAGATGGGCA GGTTATCTGG GTCAACAATA   360
CCATCATCAA TGGGAGCCAG GTGTGGGGAG GACAGCCAGT   400
GTATCCCCAG GAAACTGACG ATGCCTGCAT CTTCCCTGAT   440
GGTGGACCTT GCCCATCTGG CTCTTGGTCT CAGAAGAGAA   480
GCTTTGTTTA TGTCTGGAAG ACCTGGGGCC AATACTGGCA   520
ATTTCTAGGG GGCCCAGTGT CTGGGCTGAG CATTGGGACA   560
GGCAGGGCAA TGCTGGGCAC ACACACCATG GAAGTGACTG   600
TCTACCATCG CCGGGGATCC CGGAGCTATG TGCCTCTTGC   640
TCATTCCAGC TCAGCCTTCA CCATTACTGA CCAGGTGCCT   680
TTCTCCGTGA GCGTGTCCCA GTTGCGGGCC TTGGATGGAG   720
GGAACAAGCA CTTCCTGAGA AATCAGCCTC TGACCTTTGC   760
CCTCCAGCTC CATGACCCCA GTGGCTATCT GGCTGAAGCT   800
GACCTCTCCT ACACCTGGGA CTTTGGAGAC AGTAGTGGAA   840
CCCTGATCTC TCGGGCACTT GTGGTCACTC ATACTTACCT   880
GGAGCCTGGC CCAGTCACTG CCCAGGTGGT CCTGCAGGCT   920
GCCATTCCTC TCACCTCCTG TGGCTCCTCC CCAGTTCCAG   960
GCACCACAGA TGGGCACAGG CCAACTGCAG AGGCCCCTAA  1000
CACCACAGCT GGCCAAGTGC CTACTACAGA AGTTGTGGGT  1040
ACTACACCTG GTCAGGCGCC AACTGCAGAG CCCTCTGGAA  1080
CCACATCTGT GCAGGTGCCA ACCACTGAAG TCATAAGCAC  1120
```

FIG. 4B

```
TGCACCTGTG CAGATGCCAA CTGCAGAGAG CACAGGTATG 1160
ACACCTGAGA AGGTGCCAGT TTCAGAGGTC ATGGGTACCA 1200
CACTGGCAGA GATGTCAACT CCAGAGGCTA CAGGTATGAC 1240
ACCTGCAGAG GTATCAATTG TGGTGCTTTC TGGAACCACA 1280
GCTGCACAGG TAACAACTAC AGAGTGGGTG GAGACCACAG 1320
CTAGAGAGCT ACCTATCCCT GAGCCTGAAG GTCCAGATGC 1360
CAGCTCAATC ATGTCTACGG AAAGTATTAC AGGTTCCCTG 1400
GGCCCCCTGC TGGATGGTAC AGCCACCTTA AGGCTGGTGA 1440
AGAGACAAGT CCCCCTGGAT TGTGTTCTGT ATCGATATGG 1480
TTCCTTTTCC GTCACCCTGG ACATTGTCCA GGGTATTGAA 1520
AGTGCCGAGA TCCTGCAGGC TGTGCCGTCC GGTGAGGGGG 1560
ATGCATTTGA GCTGACTGTG TCCTGCCAAG GCGGGCTGCC 1600
CAAGGAAGCC TGCATGGAGA TCTCATCGCC AGGGTGCCAG 1640
CCCCCTGCCC AGCGGCTGTG CCAGCCTGTG CTACCCAGCC 1680
CAGCCTGCCA GCTGGTTCTG CACCAGATAC TGAAGGGTGG 1720
CTCGGGGACA TACTGCCTCA ATGTGTCTCT GGCTGATACC 1760
AACAGCCTGG CAGTGGTCAG CACCCAGCTT ATCATGCCTG 1800
GTCAAGAAGC AGGCCTTGGG CAGGTTCCGC TGATCGTGGG 1840
CATCTTGCTG GTGTTGATGG CTGTGGTCCT TGCATCTCTG 1880
ATATATAGGC GCAGACTTAT GAAGCAAGAC TTCTCCGTAC 1920
CCCAGTTGCC ACATAGCAGC AGTCACTGGC TGCGTCTACC 1960
CCGCATCTTC TGCTCTTGTC CCATTGGTGA GAACAGCCCC 2000
CTCCTCAGTG GGCAGCAGGT CTGAGTACTC TCATATGATG 2040
CTGTGATTTT CCTGGAGTTG ACAGAAACAC CTATATTTCC 2080
CCCAGTCTTC CCTGGGAGAC TACTATTAAC TGAAATAAAT 2120
ACTCAGAGCC TGAAAAAAAA TAAAAAAAAA AAAAAAAAAA 2160
AAAAAAAAAA AA                                2172
```

FIG. 5A

```
  1  MDLVLKRCLL HLAVIGALLA VGATKVPRNQ DWLGVSRQLR TKAWNRQLYP
 51  EWTEAQRLDC WRGGQVSLKV SNDGPTLIGA NASFSIALNF PGSQKVLPDG
101  QVIWVNNTII NGSQVWGGQP VYPQETDDAC IFPDGGPCPS GSWSQKRSFV
151  YVWKTWGQYW QFLGGPVSGL SIGTGRAMLG THTMEVTVYH RRGSRSYVPL
201  AHSSSAFTIT DQVPFSVSVS QLRALDGGNK HFLRNQPLTF ALQLHDPSGY
251  LAEADLSYTW DFGDSSGTLI SRALVVTHTY LEPGPVTAQV VLQAAIPLTS
301  CGSSPVPGTT DGHRPTAEAP NTTAGQVPTT EVVGTTPGQA PTAEPSGTTS
351  VQVPTTEVIS TAPVQMPTAE STGMTPEKVP VSEVMGTTLA EMSTPEATGM
401  TPAEVSIVVL SGTTAAQVTT TEWVETTARE LPIPEPEGPD ASSIMSTESI
451  TGSLGPLLDG TATLRLVKRQ VPLDCVLYRY GSFSVTLDIV QGIESAEILQ
501  AVPSGEGDAF ELTVSCQGGL PKEACMEISS PGCQPPAQRL CQPVLPSPAC
551  QLVLHQILKG GSGTYCLNVS LADTNSLAVV STQLIMPGQE AGLGQVPLIV
601  GILLVLMAVV LASLIYRRRL MKQDFSVPQL PHSSSHWLRL PRIFCSCPIG
651  ENSPLLSGQQ V
```

FIG. 5B

```
Pmel17      M-----V-----Q-----P-----VPGILLT-----LLSGQQV
ME20        M-----V-----Q-----L-----.......------------
gp100       M-----V-----Q-----L-----.......------------
cDNA25FL    M-----F-----Q-----L-----.......------------
cDNA25TR                Q-----L-----.......-----PPQWAAGLSTLI
            1    162   236   274   588         649
```

FIG. 6A

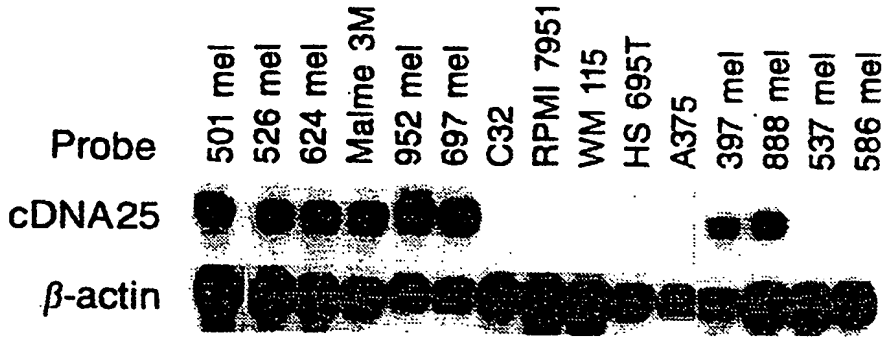


FIG. 6B

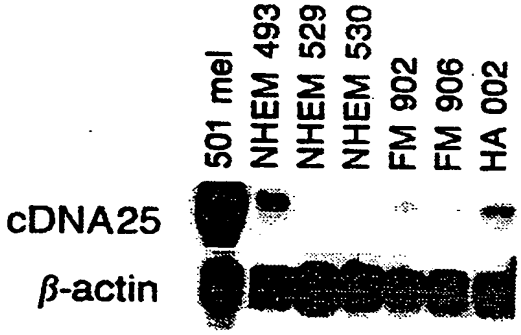


FIG. 6C

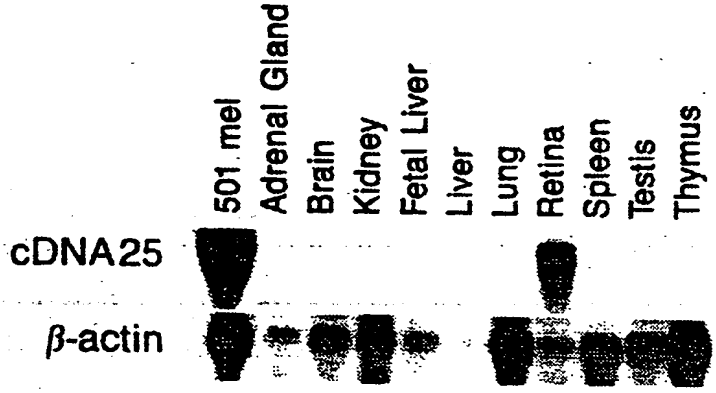


FIG. 7A

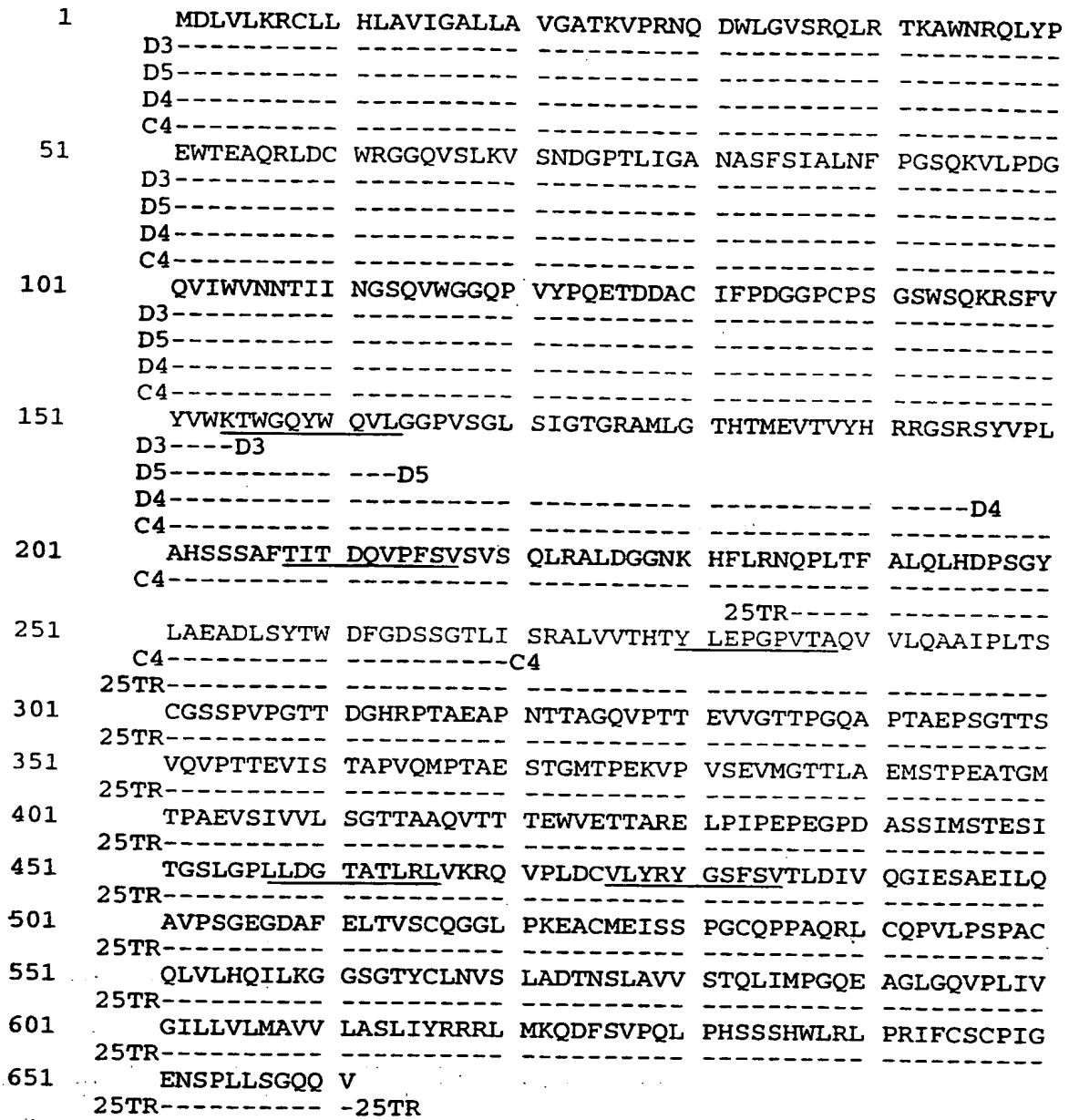
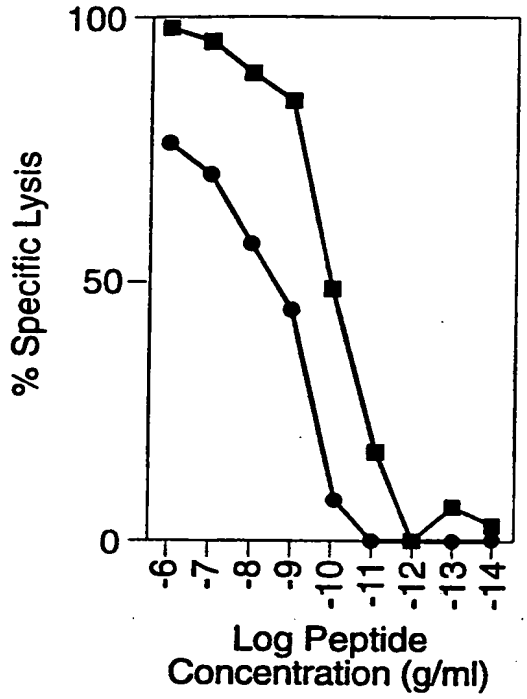

1 MDLVLKRCLL HLAVIGALLA VGATKVPRNQ DWLGVSRQLR TKAWNRQLYP
D3---------- ---------- ---------- ---------- ----------
D5---------- ---------- ---------- ---------- ----------
D4---------- ---------- ---------- ---------- ----------
C4---------- ---------- ---------- ---------- ----------
51 EWTEAQRLDC WRGGQVSLKV SNDGPTLIGA NASFSIALNF PGSQKVLPDG
D3---------- ---------- ---------- ---------- ----------
D5---------- ---------- ---------- ---------- ----------
D4---------- ---------- ---------- ---------- ----------
C4---------- ---------- ---------- ---------- ----------
101 QVIWVNNTII NGSQVWGGQP VYPQETDDAC IFPDGGPCPS GSWSQKRSFV
D3---------- ---------- ---------- ---------- ----------
D5---------- ---------- ---------- ---------- ----------
D4---------- ---------- ---------- ---------- ----------
C4---------- ---------- ---------- ---------- ----------
151 YVWKTWGQYW QVLGGPVSGL SIGTGRAMLG THTMEVTVYH RRGSRSYVPL
D3----D3
D5---------- ---D5
D4---------- ---------- ---------- ---------- -----D4
C4---------- ---------- ---------- ---------- ----------
201 AHSSSAFTIT DQVPFSVSVS QLRALDGGNK HFLRNQPLTF ALQLHDPSGY
C4---------- ---------- ---------- ---------- ----------
25TR----- ----------
251 LAEADLSYTW DFGDSSGTLI SRALVVTHTY LEPGPVTAQV VLQAAIPLTS
C4---------- ----------C4
25TR---------- ---------- ---------- ---------- ----------
301 CGSSPVPGTT DGHRPTAEAP NTTAGQVPTT EVVGTTPGQA PTAEPSGTTS
25TR---------- ---------- ---------- ---------- ----------
351 VQVPTTEVIS TAPVQMPTAE STGMTPEKVP VSEVMGTTLA EMSTPEATGM
25TR---------- ---------- ---------- ---------- ----------
401 TPAEVSIVVL SGTTAAQVTT TEWVETTARE LPIPEPEGPD ASSIMSTESI
25TR---------- ---------- ---------- ---------- ----------
451 TGSLGPLLDG TATLRLVKRQ VPLDCVLYRY GSFSVTLDIV QGIESAEILQ
25TR---------- ---------- ---------- ---------- ----------
501 AVPSGEGDAF ELTVSCQGGL PKEACMEISS PGCQPPAQRL CQPVLPSPAC
25TR---------- ---------- ---------- ---------- ----------
551 QLVLHQILKG GSGTYCLNVS LADTNSLAVV STQLIMPGQE AGLGQVPLIV
25TR---------- ---------- ---------- ---------- ----------
601 GILLVLMAVV LASLIYRRRL MKQDFSVPQL PHSSSHWLRL PRIFCSCPIG
25TR---------- ---------- ---------- ---------- ----------
651 ENSPLLSGQQ V
25TR---------- -25TR

FIG. 7B

DNA fragment	TIL 620-1	620-2	660-1	1143	1200
D3	-	-	-	-	-
D5	-	+	-	-	+
D4	-	+	-	-	+
C4	+	+	+	+	+
25TR	-	-	+	+	+

FIG. 8A


100
50
0
% Specific Lysis
-6
-7
-8
-9
-10
-11
-12
-13
-14
Log Peptide
Concentration (g/ml)

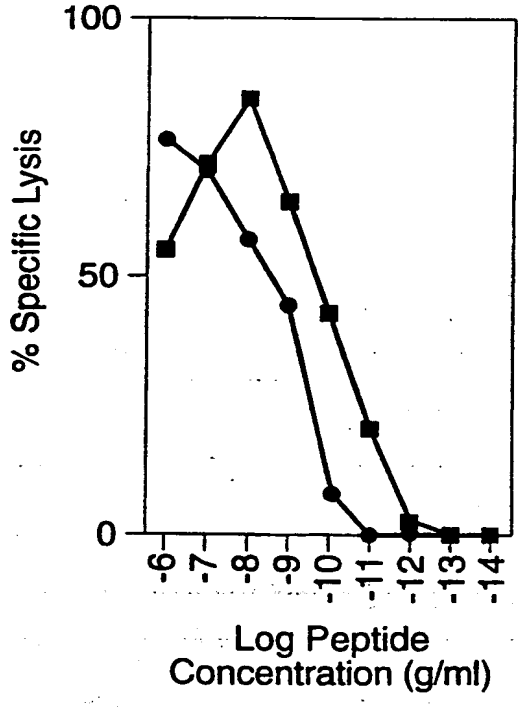
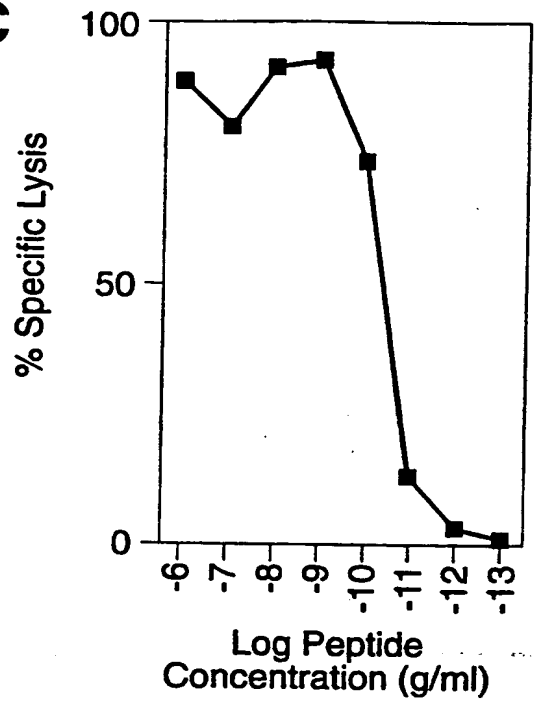

100
50
0
% Specific Lysis
-6
-7
-8
-9
-10
-11
-12
-13
-14
Log Peptide
Concentration (g/ml)

FIG. 8B

FIG. 8C


100
50
0
% Specific Lysis
-6
-7
-8
-9
-10
-11
-12
-13
Log Peptide
Concentration (g/ml)

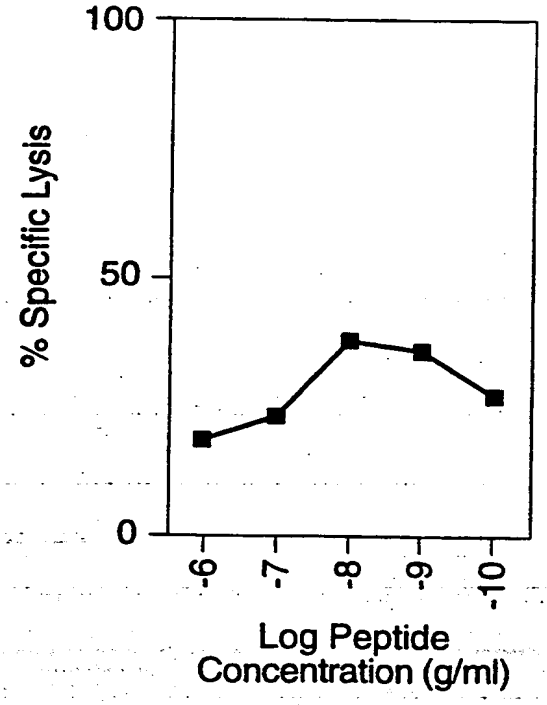

100
50
0
% Specific Lysis
-6
-7
-8
-9
-10
Log Peptide
Concentration (g/ml)

FIG. 8D